

CHOLESTEROL METABOLISM IN GUINEA PIGS

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by

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This thesis is my own work. Any collaboration with colleagues has been acknowledged.

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### SUMMARY

The studies described in this thesis have been mainly concerned with an examination of the rates of sterologogenesis and its regulation in various tissues of the guinea pig. The incorporation of radiolabelled acetate and mevalonate into total digitonin-precipitable sterols by tissue slices was used as a measure of sterologogenesis.

The rate of sterologogenesis in the liver was found to be very low. Although a diurnal variation occurred with a peak about 4 h after the commencement of the dark period, this variation was often masked by the marked differences between guinea pigs in the overall rate of hepatic sterologogenesis. The ileum showed the opposite diurnal variation in sterologogenesis to that in the liver with a peak about 4 h after the commencement of the light period. Thus depending on the time of day the rate of sterologogenesis in the ileum was 6-14 times higher than that in the liver while in the lung the rate was up to 3 times that in the liver. In contrast rats showed a higher rate of sterologogenesis in the liver than in the ileum but in both tissues the diurnal rhythm in sterologogenesis was similar with a peak during the dark period. There was no diurnal variation in the concentration of cholesterol in the plasma and tissues of guinea pigs. Similarly, there was no diurnal variation in plasma cholesterol concentration in rats.

In guinea pigs the ileum showed a higher rate of sterologogenesis than any other region of the gastrointestinal tract. Total sterol production in the ileum alone exceeded that in the liver. It was thus concluded that in the normal guinea pig the gastrointestinal tract makes a much greater

contribution to total body sterol synthesis than does the liver. Although total sterol production by the lung was less than that of the liver, the lung is quantitatively a much more important site of sterologogenesis in the guinea pig than it has been previously found to be in other species. The adrenal, testis, muscle, adipose tissue and skin showed relatively low rates of sterologogenesis and thus are probably not quantitatively important. Ageing resulted in a significant decrease in intestinal but not hepatic sterologogenesis.

Guinea pigs and rabbits showed a diurnal rhythm in feed intake with peaks of eating at the beginning and end of the light period and in the middle of the dark period. However feed intake in the dark period did not differ from that in the light period. In contrast rats showed one major peak of eating which occurred during the dark period. It was concluded that the difference in the feeding pattern of the guinea pig and rat may account for the difference in the timing of the diurnal rhythms of hepatic and intestinal sterologogenesis in these species.

The effects of diets containing added cholesterol or cholestyramine and of fasting on sterologogenesis in the liver, ileum and lung were studied in male and female guinea pigs. In males cholesterol feeding inhibited sterologogenesis in all three tissues, particularly the liver. However in females cholesterol feeding significantly decreased sterologogenesis in the ileum but not in the liver and lung although there was marked individual variation in the rate of hepatic sterologogenesis. It appeared that these guinea pigs differed markedly in their capacity to metabolise an increased dietary cholesterol load and this lead to varying



degrees of feedback inhibition of sterologogenesis in the liver. The major factor regulating sterologogenesis in the

intestine. The effect of cholestyramine feeding on sterol synthesis differed markedly between the three tissues. In both males and females sterologogenesis in the liver and lung was enhanced, the effect being particularly pronounced in the liver where the rate of sterologogenesis was increased 5- or 13-fold depending on when the animals were killed in relation to the lighting cycle. In contrast cholestyramine feeding either had no effect or increased only marginally the rate of sterologogenesis in the ileum depending on the time when the animals were killed. Cholesterol and cholestyramine feeding did not significantly affect fatty acid synthesis in the liver, ileum and lung. This showed that the effect of these treatments was specific to the pathway of sterologogenesis. Cholesterol in the guinea pig has been studied.

In guinea. Cholesterol feeding increased the concentration of cholesterol in the plasma and liver but not in the other tissues. Cholestyramine feeding had an extreme hypocholesterolaemic action but did not affect tissue cholesterol concentrations. Fatty acid concentrations were also slightly

decreased. Fasting for 24 h significantly decreased the rate of sterologogenesis in the liver, ileum and lung although the effect in the liver was greater than in the other tissues. Only in the lung was fatty acid synthesis significantly decreased. Fasting significantly increased plasma cholesterol concentration but had no effect on tissue cholesterol concentrations. Low cholesterol levels were also observed in the

concentrat. It was concluded that the low rate of hepatic sterologogenesis in the guinea pig is the result of marked

feedback inhibition by cholesterol and also possibly by bile acids. The major factor regulating sterologogenesis in the intestine and lung is probably feedback inhibition by cholesterol. It appears that intestinal sterologogenesis in the guinea pig is not regulated by feedback inhibition by bile acids. On the basis of the differences in the incorporation rates of acetate and mevalonate into sterol it was concluded that the primary rate-limiting step in hepatic and intestinal sterologogenesis in the guinea pig may occur at or before  $\beta$ -hydroxy- $\beta$ -methylglutaryl Coenzyme A reductase. Under normal conditions sterologogenesis in the liver but not in the intestine has one or more secondary points of regulation subsequent to the primary rate-limiting step.

In other studies described in this thesis the role of ascorbic acid in the regulation of various aspects of cholesterol metabolism in the guinea pig has been examined. In guinea pigs in the early stage of acute ascorbic acid deficiency there was a decrease in the rate of acetate and mevalonate incorporation into sterol in the ileum but no such effect occurred in the liver. In these guinea pigs the blood and tissue ascorbic acid concentrations were significantly decreased but there was no significant change in cholesterol concentrations. An attempt to determine the effect of dietary ascorbic acid deficiency on the activity of cholesterol 7 $\alpha$ -hydroxylase was unsuccessful. No enzyme activity could be detected in liver preparations from normal, scorbutic or cholestyramine-fed guinea pigs. The group fed the cholestyramine diet did show a significant decrease in the concentration of ascorbic acid in the liver.

Female guinea pigs fed a diet containing no added

cholesterol and supplemented with 10 times their daily vitamin C requirement for 32 weeks showed significantly higher concentrations of ascorbic acid in the blood and tissues than a corresponding group which received the normal level of ascorbic acid supplementation. However there was no effect on hepatic and intestinal sterologenesis, plasma and tissue cholesterol concentrations or on plasma triglyceride concentration. In contrast supplementation at 20 times the normal vitamin C requirement to female guinea pigs fed a diet without added cholesterol, significantly enhanced plasma cholesterol concentration. This level of supplementation also enhanced the plasma cholesterol concentration in female guinea pigs with dietary cholesterol-induced hypercholesterolaemia. In the females fed the cholesterol diet and given vitamin C initially at a level corresponding to the daily requirement and later at 20 times this requirement, the tissue ascorbic acid concentrations were significantly less than in the females fed the diet containing no added cholesterol and given the same levels of ascorbic acid supplementation.

In male guinea pigs fed a diet containing no added cholesterol, supplementation with up to 40 times the normal vitamin C requirement had no effect on plasma cholesterol concentration. Although there is strong evidence in the literature that a dietary inadequacy of vitamin C critically affects various aspects of cholesterol metabolism, the results of the present studies on the effects of high levels of ascorbic acid supplementation support the view that dietary vitamin C supplementation beyond the recommended daily allowance is ineffective in lowering plasma cholesterol concentration below normal levels.



## 1. Atherosclerosis

Atherosclerosis is a major cause of morbidity and mortality in present day society (Epstein, 1973). Despite tremendous development of research on the disease its exact aetiology is still not understood. There has however been great progress in identifying the many factors which contribute to the development of the disease.

Atherosclerosis is a pathological change in the intima of arteries characterised by focal thickenings of the intima which contain varying amounts of lipid, principally cholesterol and cholesterol esters (Duff and McMillan, 1951). It is believed that the earliest event in the development of the arterial lesion may be damage to the arterial intima which then acts as a focal point for initiating the atherosclerotic process (Eades, 1968).

### INTRODUCTION

Although the fundamental cause(s) of lesion development remain unknown, there are many risk factors which are known to predispose humans, particularly men, to develop atherosclerosis. The major risk factors are hypercholesterolaemia, hypertriglyceridaemia, hypertension, cigarette smoking, diabetes mellitus and obesity (Eades, 1968; Aronow et al., 1975). There are also many other factors which influence the pathogenesis of atherosclerosis. These include physical activity, mental stress, heredity, water hardness, hypothyroidism and age (Eades, 1968; Morris and Gardner, 1969; Strong and Eggen, 1969).

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The implication of the diet in the aetiology and pathogenesis of atherosclerosis continues to focus

on the constituents of the diet (Eades, 1968) despite evidence that in several animal species feeding frequency plays a significant role in the regulation of plasma cholesterol concentration and the induction of experimental atherosclerosis (Cohn, 1964). In humans a reduced frequency of meal intake is associated with increased plasma cholesterol concentrations (Cohn, 1964; Fábry et al., 1964).

Despite very substantial experimental and clinical evidence that dietary ascorbic acid deficiency may predispose to the development of atherosclerosis, it is not generally recognised as a risk factor in the aetiology of the disease. This is probably the product of a general unawareness that chronic latent scurvy is prevalent in many population groups. The involvement of ascorbic acid in the regulation of cholesterol metabolism and in atherosclerosis has been subjected to extensive review (Ginter, 1968; Shaffer, 1970; Ginter, 1972; Anonymous, 1973; Krumdieck and Butterworth, 1974).

## 2. The Role of Ascorbic Acid in the Regulation of Plasma and Tissue Cholesterol Concentrations and in the Pathogenesis of Atherosclerosis.

A fundamental metabolic defect in man is the absence of the enzyme L-gulonolactone oxidase in the biosynthetic pathway of ascorbic acid (Burns, 1957). This defect is also found in non-human primates, guinea pigs and a variety of other animals which thus all have a dietary requirement for vitamin C (King, 1973). A critical dietary deficiency of ascorbic acid in these species results in acute scurvy which is the only clinical



pathological condition for which cure and prevention is known to specifically require vitamin C. The incidence of acute or clinical scurvy in human populations, apart from its limited occurrence in infants (Henderson-Smart, 1972), is considered rare (Thomson, 1954; Bronte-Stewart et al., 1963). However a greater potential health risk exists in latent or subclinical scurvy which arises from a chronic dietary inadequacy of vitamin C.

The widespread incidence of latent scurvy in humans, first demonstrated about forty years ago (Yavorsky et al., 1934; Rinehart et al., 1938), remains evident in several population groups. Chronic dietary vitamin C deficiency is particularly prevalent in the elderly (Andrews et al., 1966; Brocklehurst et al., 1968; Wilson and Nolan, 1970; Booth and Todd, 1972; Silink et al., 1972), especially the elderly poor (Watkin, 1968) and also in the institutionalised young (Woodhill et al., 1974). Chronic latent hypovitaminosis C also occurs in those populations subjected to marked seasonal variations in their dietary vitamin C intake (Andrews et al., 1966; Budlovský, 1966; Kajaba and Bučko, 1968; Ginter et al., 1970b).

The significance of dietary inadequacy of vitamin C in the aetiology of hypercholesterolaemia and atherosclerosis first became apparent from clinical studies in Russia. Myasnikova (1947) showed that serum cholesterol concentrations could be lowered in hypercholesterolaemic patients by the administration of ascorbic acid. In the three decades since this study was carried out a large volume of literature on vitamin C and cholesterol

metabolism has accumulated. However many workers have used animals suffering from severe scurvy which completely masks the more subtle effects of a chronic dietary insufficiency of vitamin C. Only in recent years has the alternative approach of using animals with latent scurvy been adopted.

(a) Effects of ascorbic acid on plasma and tissue cholesterol concentrations.

The involvement of ascorbic acid in cholesterol metabolism is apparent from the changes which occur in plasma and tissue cholesterol concentrations during dietary vitamin C deficiency and by the hypocholesterolaemic action of ascorbic acid in hypercholesterolaemic animals and humans. Furthermore, changes in tissue ascorbic acid concentration often occur in cholesterol-fed animals.

In acutely scorbutic guinea pigs there is an increase in total body cholesterol (Lahiri and Banerjee, 1956; Banerjee and Singh, 1958; Banerjee and Ghosh, 1960). Although hypercholesterolaemia has been reported under such conditions (Bolker et al., 1956; Lahiri and Banerjee, 1956; Banerjee and Ghosh, 1960), some studies have shown no change in plasma cholesterol concentration (Banerjee and Singh, 1958; Ginter et al., 1965a). In guinea pigs in which the onset of a severe deficiency was delayed the plasma cholesterol concentration first increased and then declined to prescorbutic values with the onset of severe scurvy (Belavady and Banerjee, 1954). As shown by studies on severely scorbutic guinea pigs (Banerjee and Ghosh, 1961; Bobek and Ginter, 1962; Ginter et al., 1965b) and monkeys (Banerjee and Bandyopadhyay, 1965), these changes

in plasma cholesterol concentration reflect changes in both the proportion of the various lipoprotein fractions and their respective content of cholesterol. However there is wide variation in the nature of the changes observed - this probably resulted from the use of semiquantitative methods in the separation and analysis of the lipoprotein fractions.

As guinea pigs show a diminishing feed intake and a marked deterioration in physical condition after 17 days on a scorbutogenic diet, it is not surprising that during the latter stages of acute scurvy there are reversals of trends established earlier in the condition. For example, adrenal cholesterol concentration increases in the early stages of scurvy in guinea pigs (Oesterling and Long, 1951) but subsequently decreases (Oesterling and Long, 1951; Ginter et al., 1965a). The change in the cholesterol content of the adrenal is less marked than the change in concentration because the adrenal becomes enlarged in the later stages (Ginter et al., 1965a). Thus the differing reports that the cholesterol concentration of the adrenals shows no change (Baldwin et al., 1944) or decreases (Banerjee and Singh, 1958; Banerjee and Ghosh, 1960) in late scurvy could be explained by differences in the time of sampling in relation to the onset of scurvy and the changes in the size of the adrenal. In guinea pigs with advanced acute scurvy the concentration of cholesterol in intestine and testes increases and in liver and kidneys remains unchanged (Banerjee and Singh, 1958; Banerjee and Ghosh, 1960).



The many secondary effects of acute vitamin C deficiency on cholesterol metabolism are avoided by using animals with chronic latent hypovitaminosis C. In male guinea pigs with this condition, increased cholesterol concentrations in plasma and liver are observed (Ginter and Nemec, 1969; Ginter et al., 1969d, 1971a, 1972b, 1973b; Ginter, 1973; Nambisan and Kurup, 1975).

The studies on the effect of severe dietary vitamin C deficiency on plasma cholesterol concentration in primates, like those with guinea pigs, have not been definitive. Thus, although monkeys with severe scurvy showed reduced plasma cholesterol concentrations, repletion with ascorbic acid did not reverse this condition (Banerjee and Bal, 1959; Banerjee and Bandyopadhyay, 1965). In baboons serum cholesterol was unaffected by either high oral doses of vitamin C or by the withdrawal of all dietary vitamin C (Kotzé et al., 1974). In humans severe vitamin C deficiency was associated with a decrease in serum cholesterol concentration which was reversed on repletion with vitamin C (Tolmachev, 1934; Bronte-Stewart et al., 1963; Hodges et al., 1971). However these decreases were probably not specifically produced by vitamin C deficiency. Thus in a study of clinical scurvy in Bantus, Bronte-Stewart et al. (1963) indicated that the diet of the subjects was altered to counter general malnutrition before initial observations on serum cholesterol concentrations were made. Similarly the study of experimental scurvy in humans by Hodges et al. (1971) is of limited value because the diets contained no cellulose and breakdown of the steroid nucleus of cholesterol in the gut is known to occur on such diets (DenBesten et al., 1970).

Many clinical trials have been undertaken in which subjects have received vitamin C at doses at least an order of magnitude greater than those necessary to prevent clinical scurvy. It has generally been found that vitamin C lowers plasma cholesterol concentration in hypercholesterolaemic human subjects (Myasnikova, 1947; Tiapina, 1952; Sedov, 1956; Fedorova, 1960; Catelli et al., 1961; Sokoloff et al., 1966; Ginter et al., 1970b) but has no effect on those with normal plasma cholesterol concentrations (Anderson et al., 1958; Sokoloff et al., 1966; Crawford et al., 1975). There are however other reports showing that vitamin C supplementation to hypercholesterolaemic subjects either has no effect on plasma cholesterol concentration (Samuel and Shalchi, 1964; Peterson et al., 1975) or increases it (Spittle, 1971). These differences in findings may be related to differences between the trials in the ascorbic acid status of the subjects before the commencement of ascorbic acid supplementation. Unfortunately most of these clinical studies were carried out on only a small number of subjects and did not include measurements of blood ascorbic acid concentration.

The finding that vitamin C has no effect on normocholesterolaemic subjects, is consistent with the results of a study by Elwood et al. (1970) which found no association between plasma ascorbic acid and serum cholesterol concentrations. A more recent study on a much smaller number of people showed a positive correlation between the levels of serum cholesterol and both plasma and leucocyte ascorbate (Gatenby Davies and Newson, 1974).

However the statistical treatment of the data in this study is of doubtful validity.

Seasonal variation in vitamin C intake is an important factor in the aetiology of latent scurvy in humans. A chronic dietary insufficiency of vitamin C during the winter and spring months has been demonstrated (Andrews et al., 1966; Budlovský, 1966; Kajaba and Bučko, 1968; Ginter et al., 1970b). Although one study has shown no seasonal effect on plasma cholesterol concentration in humans, (Fuller et al., 1974), others (Keys et al., 1958; Thomas et al., 1961; Tochowicz et al., 1962; McDonough and Hames, 1967; Fyfe et al., 1968; Kajaba and Bučko, 1968; Ginter et al., 1970b) have demonstrated a distinct hypercholesterolaemia during the period of the year when the vitamin C intake becomes sub-optimal. In one of these studies blood ascorbic acid concentration was also measured and found to be inversely correlated with plasma cholesterol concentration (Kajaba and Bučko, 1968). Similarly in rats the seasonal variation in plasma cholesterol concentration is related to the variation in adrenal ascorbic acid content (Thorp and Waring, 1962).

Cholesterol feeding increases the vitamin C requirement of the guinea pig (Ginter and Zloch, 1972) and when prolonged, reduces the ascorbic acid concentration of tissues (Ginter, 1970) - there being a significant negative correlation between the concentration of cholesterol and ascorbic acid in liver, adrenal and small intestine but not in plasma (Ginter et al., 1969a). Cholesterol feeding in rabbits and rats, species which synthesise ascorbic acid (Horowitz and King, 1953; Harris et al., 1956), results in



an increased accumulation of vitamin C in the tissues and excretion in the urine indicating an enhancement of ascorbic acid biosynthesis (Ginter, 1970; Novitskii, 1971). Conversely dietary cholesterol-induced hypercholesterolaemia in rabbits (Sokoloff et al., 1967; Ginter et al., 1970a), rats (Sokoloff et al., 1967) and pigs (Hutagalung et al., 1969) is reduced by supplementation with vitamin C. The cholesterolaemic response of rabbits to dietary cholesterol shows high individual variation (Novitskii, 1971; Roberts et al., 1974). The lower cholesterolaemic response in some rabbits is associated with a greater ability to augment the biosynthesis of ascorbic acid in response to the increased dietary load of cholesterol (Novitskii, 1971). The seasonal variation in the hypocholesterolaemic action of supplementary dietary ascorbic acid in cholesterol-fed rabbits (Zaitsev et al., 1964) may have been due to the variation in the ascorbic acid content of the non-supplemented diet.

(b) Effects of ascorbic acid on atherosclerosis.

Studies by Myasnikova (as reported by Myasnikov, 1958), and also those by Myasnikov (1950, 1954) showed that ascorbic acid ameliorates the development of experimental atherosclerosis and cholesterolaemia in rabbits. These findings were confirmed in clinical trials in Russia in later years (Sedov, 1956; Fedorova, 1960). Vital statistics for England and Wales show a dicyclic seasonal variation in deaths from myocardial infarction with peaks during the winter and spring (Dunnigan et al., 1970) and it has been suggested that these peaks coincide with periods of minimum consumption of vitamin C (Spittle,

1972). More recently a correlation analysis between the standardised mortality ratios for various causes of death and the intake of a number of nutrients in different regions of England and Wales in different years has shown strong negative correlations between vitamin C intake and mortality from ischaemic heart disease (-0.49) and cerebrovascular disease (-0.68) (Knox, 1973). Such statistical correlations however do not necessarily prove a cause-and-effect relationship (Fraser, 1975).

The inference that high intakes of vitamin C reduce the incidence of atherosclerosis is supported by the results of many experimental studies. In guinea pigs fed an atherogenic diet the development of atheroma was directly related to vitamin C intake - the most advanced lesions occurred in the group which received the lowest intake (Ginter et al., 1969a). In rabbits, vitamin C supplementation ameliorated the severity of dietary cholesterol-induced atherosclerosis (Myasnikov, 1950, 1954; Chakravarti et al., 1957; Myasnikov, 1958; Zaitsev et al., 1964, 1965; Sokoloff et al., 1967; Ginter et al., 1970a) although some studies demonstrated no such effect (Flexner et al., 1941; Pool et al., 1971). In rats receiving a diet containing no added cholesterol, dietary supplementation with vitamin C markedly decreased the cholesterol and phospholipid concentration of the aorta (Nambisan and Kurup, 1974).

Ascorbic acid may influence the pathogenesis of atherosclerosis not only by an effect on plasma cholesterol concentration but also on plasma triglyceride concentration and on the integrity of the vascular wall. Sokoloff et al.

(1966) demonstrated a 50-70% reduction in plasma triglyceride concentration in patients receiving ascorbic acid supplementation and this was associated with a marked stimulation of plasma lipoprotein lipase activity. In guinea pigs fed an atherogenic diet, high doses of vitamin C reduced the triglyceride concentration in the plasma, liver and aorta but the effect on lipoprotein lipase activity in various tissues was not uniform (Nambisan and Kurup, 1975). In human arteries those segments with higher concentrations of ascorbic acid had a lower incidence and severity of atheroma (Willis and Fishman, 1955), indicating that ascorbic acid regulates arterial wall structure and function. Spittle (1971) has suggested that the mobilisation of cholesterol from the arterial wall by an increased intake of ascorbic acid in the diet could account for a reported increase in the plasma cholesterol concentration. However the magnitude of the reported increase cannot be explained by such a mobilisation (Morin, 1972). It has also been suggested that ascorbic acid decreases the development of experimental atherosclerosis in rabbits by decreasing the permeability of the aortic wall to cholesterol (Zaitsev et al., 1964).

The mechanism by which ascorbic acid could probably exert its greatest influence on the structure of the arterial wall is through its effect on collagen formation where it plays an essential role in the hydroxylation of proline. Acutely scorbutic guinea pigs have been shown to develop lesions in the aortic intima such as the separation of endothelial cells and a depletion of sub-endothelial collagen (Gore et al., 1965). Such



lesions which could be produced in guinea pigs without hypercholesterolaemia (Willis, 1953) and which could be readily reversed in the early stages by ascorbic acid therapy (Willis, 1957), were considered a direct result of a disturbance in the formation of the intimal ground substance (Willis, 1953).

The importance then of dietary ascorbic acid deficiency in the aetiology and pathogenesis of atherosclerosis is two-fold. Firstly, the changes which occur in the integrity of the vascular wall may be involved in initiating the atherosclerotic process. Secondly the hypercholesterolaemia resulting from chronic latent scurvy may contribute to the development of the lesion. The mechanisms by which dietary ascorbic acid deficiency leads to elevated plasma and liver cholesterol concentrations are discussed in a later section.

### 3. Animal Models for the Study of the Role of Ascorbic Acid in Atherosclerosis and the Control of Cholesterol Metabolism.

Controlled studies on the pathogenesis of atherosclerosis in humans are almost impossible because of man's long life span, the slow rate of lesion development and the many and varying factors which contribute to the development of lesions. These problems are compounded by the difficulty in precisely delineating the time of initiation of atherogenesis and of evaluating the severity of the disease in the individual living human subject (Getz et al., 1969). As a result of these difficulties several animal models of the disease have been developed. The advantages and disadvantages of these various animal models

have been extensively reviewed (Clarkson, 1963; Kritchevsky, 1969; Clarkson, 1972). However these review articles have not evaluated the various models for specifically examining the role of ascorbic acid in atherosclerosis.

The rabbit has been used more extensively than any other species as a model for studies relating vitamin C to the pathogenesis of atherosclerosis (Flexner et al., 1941; Myasnikov, 1950, 1954; Chakravarti et al., 1957; Myasnikov, 1958; Zaitsev et al., 1964, 1965; Sokoloff et al., 1967; Novitskii, 1969; Ginter et al., 1970a; Novitskii, 1971; Pool et al., 1971) probably because it was the first in which dietary-induced hypercholesterolaemia (Pribram, 1906) and atherosclerosis (Anitschkow, 1912) were reported. However it is held by some workers that some aspects of experimental atherosclerosis in rabbits resemble the human condition only superficially (Clarkson, 1972). Furthermore the rabbit, unlike the human, synthesises ascorbic acid and, on cholesterol feeding, increases the rate of synthesis of the vitamin to compensate for the increased requirement for vitamin C - this response shows marked individual variation (Novitskii, 1971). Thus although studies with the rabbit have generally supported the evidence for a role of vitamin C in cholesterol metabolism and atherosclerosis, its usefulness as a model for future work is limited.

The ideal model for this work would be one which is unable to synthesise ascorbic acid. Although this restricts the selection essentially to the various non-human primates and the guinea pig, there are other species unable to synthesise ascorbic acid, such as the Indian

fruit-eating bat and various birds including the red-vented bulbul (King, 1973). These may be useful in specific circumstances.

It is widely held that atherosclerosis in non-human primates may be particularly relevant to the human disease; it is thus surprising that the relationship between ascorbic acid and atherosclerosis has not been examined in these species. The required level of deficiency as well as the speed and severity of onset of the disease are obvious factors to investigate.

Although the guinea pig does not develop atherosclerosis under normal conditions (Weinberg, 1908; Saphir, 1925), the disease can be induced experimentally. As already discussed scorbutic guinea pigs develop atherosclerotic lesions in the aortic intima (Gore et al., 1965). Such lesions appear even though the animals may not be hypercholesterolaemic (Willis, 1953). Thus the guinea pig would be useful for further examining the role of dietary ascorbic acid deficiency in initiating atherosclerosis. Most studies, contrary to that of Cook and McCullagh (1938), have shown that cholesterol-fed guinea pigs develop atherosclerosis (Bailey, 1915; Anitschkow, 1922; Schöenheimer, 1924; Altschul, 1950; Bernick et al., 1962; Babala and Ginter, 1968; Ginter et al., 1968a, 1969a). The development of lesions occurs slowly and at plasma cholesterol concentrations closely paralleling those of hypercholesterolaemic humans (Babala and Ginter, 1968; Ginter et al., 1968a). Thus there is scope for further use of the cholesterol-fed guinea pig as a model for examining the effectiveness of ascorbic



acid in ameliorating atherosclerosis and hypercholesterolaemia. The high individual variation between guinea pigs with dietary-induced cholesterol atherosclerosis, considered a limitation of this model (Clarkson, 1963), might be overcome if strains of guinea pigs with a more predictable cholesterolaemic response could be selected. This has been achieved with the rabbit (Roberts et al., 1974) and squirrel monkey (Clarkson et al., 1971). Guinea pigs maintained under conditions where they naturally develop latent scurvy during the winter and spring months (Ginter et al., 1972a), could be used as models for examining the impact of seasonal variations in vitamin C intake on the pathogenesis of atherosclerosis.

The baboon and rhesus monkey are the only non-human primates that have been used for studying the role of ascorbic acid in specific aspects of cholesterol metabolism (Banerjee and Bandyopadhyay, 1965; Kotzé et al., 1974; Weight et al., 1974). The particular value of the baboon is that its lipoproteins are similar to those in man (Kritchevsky et al., 1965), in contrast to the absence of HDL in the guinea pig - except when fed cholesterol (Puppione et al., 1971). Nevertheless, the guinea pig has over-riding advantages in cheapness and the rapidity with which its ascorbic acid can be depleted; half-life of ascorbic acid in the guinea pig is about 4 days (Burns et al., 1956; Ginter et al., 1971b). Although the pathway of ascorbic acid catabolism is different in guinea pigs and humans (Hellman and Burns, 1955; Burns et al., 1956), the quantity and relative distribution of ascorbic acid in guinea pig tissues is similar to that in human tissues (Yavorsky et al., 1934).

Chronic deficiency of ascorbic acid in guinea pigs provides a better model than acute deficiency where, in the later stages animals develop anorexia, weight loss, negative nitrogen balance, bleeding and anaemia (Chatterjee, 1967a). Ginter and his group have developed a particularly suitable model of chronic latent hypovitaminosis C in guinea pigs (Ginter et al., 1968b). Animals are fed a scorbutogenic diet for 14 days and are then maintained on 0.5 mg ascorbic acid per day. This regime results in tissue ascorbic acid concentrations comparable with those in acute scurvy. However only in males does this chronic deficiency lead to increased cholesterol concentrations (Ginter and Ondreička, 1971).

Although the vitamin C requirement of guinea pigs is given as 16 mg/kg body weight/day (Reid and Bieri, 1972), Williams and Deason (1967) have shown a 20-fold variation in the requirement of individual guinea pigs. This is possibly related to individual differences in the rate at which ascorbic acid is catabolised. It may also indicate varying abilities to synthesise ascorbic acid. There is evidence that a small number of female guinea pigs can synthesise ascorbic acid (Odumosu and Wilson, 1973) but this is not accepted universally (Barnes et al., 1973; Jones et al., 1973). This individual variation could be used to advantage if strains of guinea pigs could be selected for differences in vitamin C requirement.

As will be shown in a later section the guinea pig has been used widely in studies of the role of ascorbic acid in various aspects of cholesterol metabolism. The cholesterol-fed guinea pig has also been a particularly

valuable model for familial lecithin:cholesterol acyltransferase deficiency because cholesterol-fed guinea pigs show abnormalities in plasma lipoproteins which resemble those found in the familial condition in man (Glomset and Norum, 1973). Although the guinea pig has been used widely in these specific studies, there is much less known about the control of cholesterol metabolism in the normal guinea pig than in other species such as the rat. This applies particularly to the synthesis of cholesterol and its regulation in various tissues.

#### 4. Regulation of Cholesterol Metabolism.

##### (a) General Aspects.

The regulation of cholesterol metabolism has been extensively reviewed by Dietschy and Wilson (1970a,b,c) as well as by Myant (1970) and Nestel (1970) although the latter reviews are concerned mainly with cholesterol metabolism in humans. Other reviews are concerned with specific aspects of cholesterol metabolism such as the regulation of cholesterol synthesis (Siperstein, 1970; Romsos and Leveille, 1974; Rodwell et al., 1976) and the role of bile acids in the overall control of steroid metabolism (Wilson, 1972b). As these articles present a detailed account of the absorption, synthesis, catabolism and excretion of cholesterol, the present review will describe these processes only in general terms.

##### (i) Cholesterol absorption.

The major steps in the absorption of cholesterol have been described by Dietschy and Wilson (1970b). Cholesterol in the intestinal lumen consists of a mixture of free and esterified cholesterol derived from either the



diet or from various endogenous sources such as the bile, other intestinal secretions and desquamated mucosal cells. Cholesteryl ester is hydrolysed to free cholesterol by bile salt activated pancreatic cholesteryl ester hydrolase. In the presence of sufficient concentrations of bile acids, monoglycerides and fatty acids, free cholesterol is in turn solubilised in mixed micelles and in this form is brought into contact with the brush borders of the intestinal absorptive cells. Once absorbed the cholesterol mixes with the intracellular pool of unesterified cholesterol some of which is derived from synthesis by the epithelial cell. Most of the cholesterol is then esterified, incorporated into chylomicra and released into the intestinal lymph. The movement of chylomicra from the mucosa into the lymph is probably the rate-limiting step in cholesterol absorption (Sylvén and Borgström, 1968). Cholesterol contained in lymph chylomicra subsequently enters the blood stream via the thoracic duct. Triglyceride is rapidly removed from the chylomicra in the extrahepatic tissues by lipoprotein lipase and the cholesterol eventually reaches the liver contained in chylomicron remnant particles (Redgrave, 1970).

Maximal absorption of cholesterol occurs in the proximal jejunum (Borgström, 1960). The capacity for cholesterol absorption in species such as the dog, rat and rabbit is far greater than in humans (Dietschy and Wilson, 1970b). The absorption of cholesterol by the intestine of guinea pigs is intermediate between that of rabbits and rats (Cook and Thomson, 1951). As a result of the much higher rate of cholesterol absorption these

animal species become hypercholesterolaemic when fed diets containing added cholesterol. Species differences in the rate of cholesterol absorption may be the result of variations in the rate of movement of cholesterol from the mucosa into the lymph (Dietschy and Wilson, 1970b). The feeding of a high fat diet increases cholesterol absorption (Wilson, 1962) whereas biliary diversion has the opposite effect (Siperstein et al., 1952).

(ii) Cholesterol transport.

Lipid is transported in the blood in the form of lipoproteins. On the basis of various physical properties lipoproteins have been divided into five classes; chylomicra, very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL) and very high density lipoproteins (VHDL). These show different protein and lipid compositions. Most of the triglyceride is transported in the chylomicra and VLDL whereas most of the cholesterol is transported in the LDL and HDL. Much of the cholesterol is present as cholesteryl ester which is produced in the plasma by the enzyme lecithin:cholesterol acyltransferase. Plasma lipoproteins are synthesised in the liver and intestine. Most lipoprotein catabolism probably occurs in the liver. However the initial stage of chylomicra catabolism takes place in the extrahepatic tissues. The structure and metabolism of lipoproteins have been recently reviewed by Jackson et al. (1976).

(iii) Cholesterol synthesis.

The balance studies of Schöenheimer and Breusch (1933) provided the first definitive evidence that de novo synthesis of cholesterol occurred in intact animals.

Bloch and Rittenberg (1942) later demonstrated that acetate represented the major carbon source for cholesterol synthesis in animal tissues. It was subsequently shown that most tissues in the body had the capacity to synthesise cholesterol (Srere et al., 1950). Since these early studies the details of the pathway of cholesterol synthesis and the intracellular location of enzymes in the pathway have been elucidated (Dennick, 1972).

The rate-limiting enzyme in cholesterol synthesis in rat liver is  $\beta$ -hydroxy- $\beta$ -methylglutaryl Coenzyme A (HMG CoA) reductase (Siperstein and Fagan, 1966; Slakey et al., 1972). The enzyme is located in the microsomes and catalyses the reduction of HMG CoA to mevalonate. Studies with rats also indicate that HMG CoA reductase may be the rate-limiting enzyme in cholesterol synthesis in the intestine (Dietschy, 1968). There is also evidence to suggest that under certain conditions there may be other secondary points of regulation in liver cholesterol synthesis which occur before (White, 1972) and after (Gould and Swyryd, 1966; Slakey et al., 1972) HMG CoA reductase. There is no information concerning the identity of the rate-limiting enzyme (or enzymes) in sterologogenesis in guinea pig tissues.

*Rates of cholesterol synthesis in various tissues.* The relative rates of sterologogenesis in various tissues have been examined in several species. Most of these studies have involved the measurement of the incorporation rate of radiolabelled acetate into cholesterol or, less specifically, into total digitonin-precipitable sterols by tissue slices. Such studies with



rat tissues have demonstrated that the liver and ileum show the highest rates of cholesterol synthesis - the liver being quantitatively a more important site of synthesis than the intestine (Dietschy and Siperstein, 1967). In monkeys the relative rates of hepatic and intestinal cholesterol synthesis vary markedly with the species, although the liver consistently shows a higher rate of cholesterol synthesis than does the intestine (Dietschy and Wilson, 1968; Corey and Hayes, 1974). It has also been shown that in baboons the rate of cholesterol synthesis in the liver exceeds that in the ileum (Wilson, 1972a). In the rat (Dietschy and Siperstein, 1967) and squirrel monkey (Dietschy and Wilson, 1968) more than 90% of total body cholesterol synthesis occurs in the liver and gastrointestinal tract.

*In vivo* The relatively lower rates of cholesterogenesis in the intestine of the rat, monkey and baboon are in marked contrast to those in the human in which the rate in the ileum is about 4 times that in the liver (Dietschy and Gamel, 1971). The results of studies on the relative rates of sterol synthesis in the guinea pig suggest that this species may show similar tissue differences in the rates of sterol synthesis to those in the human. Thus Schwenk et al. (1955), in a study with guinea pigs found that the incorporation rate of labelled acetate into sterols in vivo by the gastrointestinal tract was approximately 10 times higher than that shown by the liver. A more recent study by Swann and Siperstein (1972) demonstrated that in guinea pigs, the rate of sterol synthesis in vitro in the liver was very low relative to that in the intestine.

Although the measurement of sterol synthesis in guinea pig liver and intestine in vitro and in vivo has given similar results this has not been found in studies with rats. Under in vivo conditions the rate of sterologogenesis in the intestine exceeds that in the liver (Edwards et al., 1972; Chevallier and Magot, 1975).

However such in vivo studies may not take into account the possibility that labelled sterol synthesised in the liver enters the bile and subsequently is reabsorbed by the intestinal wall. Thus it would not be possible in these studies to distinguish between sterol synthesised by the intestine and that derived originally from the liver. As the rate of hepatic sterologogenesis in the rat greatly exceeds that in the guinea pig (Beher et al., 1963) the study of the rates of intestinal sterologogenesis under in vivo conditions would be less conclusive in rats than in guinea pigs.

In the rat (Dietschy and Siperstein, 1967), squirrel monkey (Dietschy and Wilson, 1968) and human (Dietschy and Gamel, 1971) sterologogenesis in the liver, intestine and various other tissues is considered a direct measure of cholesterologenesis because the principal sterol produced is cholesterol. While this also applies to sterol synthesis in the liver of the guinea pig, it cannot be applied to intestinal sterologogenesis in this species because the principal sterols produced are lathosterol and 7-dehydrocholesterol (Ockner and Laster, 1966).

*Diurnal variation in cholesterol synthesis.* Rats fed ad libitum and maintained under controlled lighting with 12 h of light and 12 h of dark, show a marked diurnal

variation in cholesterol synthesis in the liver which peaks about the middle of the dark period (Back et al., 1969; Horton et al., 1970; Edwards et al., 1972). The small intestine also shows the same rhythm in cholesterol synthesis although the amplitude is less than in the liver (Edwards et al., 1972). These rhythmic changes in the incorporation of acetate into cholesterol are paralleled exactly by changes in the activity of HMG CoA reductase (Shefer et al., 1972b). Although the hamster shows a similar diurnal variation in hepatic and intestinal cholesterologenesis to the rat (Ho, 1975), the peak of the rhythm in mouse liver occurs towards the end of the light period (Kandutsch and Saucier, 1969). There appears to be no information concerning diurnal variations in sterologogenesis in the tissues of other species. Such information would be particularly useful in the study of hepatic sterologogenesis in guinea pigs because the rate is normally very low and would therefore be most appropriately measured at the peak of the diurnal rhythm. The significance of determining the nature and extent of such diurnal fluctuations is also, partly, that total daily sterol synthesis in any organ can be more accurately quantitated. In addition the extent to which treatments such as cholestyramine feeding alter the rate of cholesterol synthesis depends on when the animals are killed in relation to the normal diurnal rhythm in cholesterologenesis (Horton et al., 1970; Shefer et al., 1972b; Weis and Dietschy, 1975).

The regulation of the diurnal rhythm of cholesterol synthesis and HMG CoA reductase activity in rat liver has been extensively studied. This work has been



reviewed recently by Romsos and Leveille (1974) and Rodwell et al. (1976). Studies by Edwards et al. (1972) and Dugan et al. (1972) have examined the effect of cyclic changes in lighting and feed intake on the rhythm. Rats fed ad libitum and maintained under 12 h of light and 12 h of dark consumed most of their feed during the dark period. In these rats the peak of the diurnal rhythm in cholesterol synthesis and reductase activity occurred about the middle of the dark period. However in rats adapted to eating for only a few hours during the light period, the peak of the rhythm occurred several hours later and within the light period. This showed that the cyclic pattern of feed intake and not the lighting cycle per se regulates the timing of the rhythm. Other studies have shown that the rhythm persists in fasted rats (Hamprecht et al., 1969; Dugan et al., 1972; Hickman et al., 1972; Weis and Dietschy, 1975) although the rate of cholesterol synthesis is greatly decreased. This demonstrates that the occurrence of the rhythm is independent of feed intake although its magnitude and timing are not.

Several hormones have been shown to influence the activity of HMG CoA reductase in rat liver. Insulin and triiodothyronine, together but not alone, stimulate reductase activity. However other hormones such as glucagon and hydrocortisone inhibit the stimulatory effect of insulin and triiodothyronine (Dugan et al., 1974; Huber et al., 1974). The inhibitory effect of hydrocortisone reported by Dugan et al. (1974) contrasts to the results of studies by Nervi and Dietschy (1974) which showed that adrenalcorticosteroids do not directly influence hepatic cholesterogenesis in the rat.

Dugan et al. (1974) have suggested that cyclic changes in feed intake may regulate the timing of the rhythm in HMG CoA reductase activity by regulating the relative concentrations of the stimulatory and inhibitory hormones. There is some evidence to support this suggestion. Thus Kaul and Berdanier (1972) have reported that in rats fed ad libitum there is a diurnal variation in plasma insulin concentration which is similar to that in HMG CoA reductase activity. In addition, studies with humans have shown that following ingestion of carbohydrate, insulin secretion increases whereas glucagon secretion decreases (Unger et al., 1963).

The major limitation of studying the relationship between feeding pattern and the regulation of cholesterol synthesis in rats is that this species has a different feeding pattern to the human. Rats show only one major peak of eating during a 24-h period whereas the feeding pattern of humans is characterised by multiple peaks of eating. Thus the hormonal-mediated changes in cholesterol synthesis which occur in rats in response to feeding might be much more complex in humans and other species with a feeding pattern in which there are several periods of increased eating activity during a 24-h period. However before this possibility can be examined the feeding patterns of other laboratory animals such as the guinea pig and rabbit must be studied. The significance of determining the precise nature of the interaction between feeding and the levels of hormones which regulate the rate of cholesterol synthesis is that in animals and humans a decrease in meal frequency leads to enhanced plasma

cholesterol concentration (Cohn, 1964; Fábry et al., 1964).

*Regulation of cholesterol synthesis.* As the liver and intestine are the major sites of cholesterol synthesis the regulation of cholesterologenesis in these tissues has been widely studied. Evidence from studies with several mammalian species including humans suggests that hepatic and intestinal cholesterologenesis are controlled differently (Dietschy and Wilson, 1970a). In the rat (Dietschy and Siperstein, 1965, 1967; Cayen, 1969), squirrel monkey (Dietschy and Wilson, 1968) and man (Bhattathiry and Siperstein, 1963; Dietschy and Gamel, 1971), cholesterol feeding markedly inhibits hepatic cholesterologenesis while having relatively little effect on cholesterol synthesis in the intestine. This indicates that cholesterol synthesis in the liver but not in the intestine is under direct feedback inhibition by cholesterol. In contrast biliary diversion in the rat (Dietschy, 1968; Weis and Dietschy, 1969) and squirrel monkey (Dietschy and Wilson, 1968) has a much greater stimulatory effect on intestinal cholesterol synthesis than it has on cholesterol synthesis in the liver. The increase in cholesterol synthesis in rat intestine can be prevented by the infusion of whole bile and of pure bile acids such as taurocholate into the intestinal lumen of bile duct cannulated animals whereas cholestyramine treated bile is not capable of preventing the enhanced cholesterol synthesis (Dietschy, 1968). Cholestyramine is a resin which binds bile acids and when fed to animals results in increased faecal excretion of bile acids (Huff et al., 1963). In patients with biliary obstruction a marked increase in cholesterol synthesis in



the intestine has been reported (Dietschy and Gamel, 1971). Together, these findings suggest that intestinal sterologogenesis is regulated primarily by direct feedback inhibition by bile acids.

The feeding of a high cholesterol diet to rats for 1 week was shown to result in a small but significant depression of cholesterol synthesis in the intestine (Dietschy, 1968). However this effect was attributed to the increased concentration of bile acids in the intestinal lumen and not to direct feedback inhibition by cholesterol. Thus it appears that dietary cholesterol influences intestinal sterologogenesis only indirectly by an effect on bile acid synthesis.

Other studies with rats have shown that cholesterol synthesis in the liver is decreased by feeding bile acids (Beher et al., 1963; Back et al., 1969) and is increased by biliary diversion (Weis and Dietschy, 1969) and cholestyramine feeding (Huff et al., 1963). Although these findings suggest that bile acids directly regulate cholesterol synthesis in the liver it is considered that the enhanced rate of cholesterol synthesis results because of an effect of these treatments on cholesterol absorption (Weis and Dietschy, 1969). There is however other evidence which strongly implies a direct role of bile acids in regulating cholesterol synthesis in the liver. Thus Wilson (1972a) showed that in baboons the enhancement of hepatic cholesterol synthesis that followed ileal diversion was not suppressed even to the normal range by a high cholesterol diet, despite the fact that the absorption of dietary cholesterol was unimpaired. Similarly Hamprecht

et al. (1971b) demonstrated that feeding cholic acid to rats with diversion of thoracic duct lymph significantly decreased hepatic HMG CoA reductase activity. Thus although the amount of cholesterol reaching the liver from the intestine may be the major factor which directly regulates hepatic cholesterologenesis, the possibility that bile acids also exert some direct regulatory effect cannot be excluded.

Another factor which has been shown to influence the rate of cholesterol synthesis is the level of caloric intake. In rats (Dietschy and Siperstein, 1967) and squirrel monkeys (Dietschy and Wilson, 1968) fasting has a much greater inhibitory action on cholesterol synthesis in the liver than in the intestine. However in humans intestinal cholesterologenesis is inhibited by fasting to a much greater extent than in the rat and squirrel monkey (Dietschy and Gamel, 1971).

The control of cholesterol synthesis, particularly in the liver, is complex not only because of the diversity of environmental factors which alter the rate of cholesterol synthesis but also because the effects of these factors can be superimposed. Thus in rats the diurnal rhythm in liver cholesterol synthesis persists in animals in which the rate of cholesterologenesis has been either enhanced by feeding cholestyramine or  $\beta$ -sitosterol or decreased by cholesterol feeding or fasting (Weis and Dietschy, 1975). These findings suggest that various environmental factors influence the rate of hepatic sterol synthesis through several different effectors rather than through a single mediator. Although the identity of the effectors may vary

in different physiological circumstances it is certain that all effectors regulate the activity of HMG CoA reductase. Thus the changes in the rate of acetyl CoA incorporation into cholesterol which occur in the liver during various experimental manipulations such as the feeding of cholesterol or cholestyramine, or fasting, are paralleled exactly by changes in the activity of the reductase (Dietschy and Brown, 1974).

The identity of the effectors which are operative under various circumstances is not known and the mechanisms by which these effectors regulate the activity of HMG CoA reductase are poorly understood. In rats the inhibition of cholesterol synthesis in the liver produced by cholesterol feeding (Gould and Swyryd, 1966) or by the intravenous administration of lipoproteins (Nervi and Dietschy, 1975) is invariably associated with an increase in the cholesteryl ester content of the liver. However the diurnal variation in liver cholesterol synthesis is not associated with any change in hepatic cholesteryl ester content (Nervi et al., 1975). Although these findings imply that cholesteryl ester has a role in the regulation of HMG CoA reductase activity during feedback inhibition, the actual effector is not necessarily cholesteryl ester but possibly a small pool of unesterified cholesterol or an unrecognised metabolic product of cholesterol (Nervi and Dietschy, 1975).

The mechanisms by which HMG CoA reductase activity is regulated appear to vary with different physiological circumstances. Thus evidence suggests that diurnal changes in reductase activity involve changes in



the rate of new enzyme synthesis (Edwards and Gould, 1972) whereas the decrease in enzyme activity produced by cholesterol feeding may result because of end-product inhibition and/or an increase in the rate of enzyme degradation (Edwards and Gould, 1974).

Most studies concerned with elucidating the fine aspects of the regulation of cholesterol synthesis have been carried out using rat liver. However in recent years another line of research has developed which has specifically examined the role of lipoproteins in the regulation of HMG CoA reductase activity in cultured human fibroblasts (Brown and Goldstein, 1973, 1974; Goldstein and Brown, 1974; Brown et al., 1974). These studies have demonstrated that in patients with familial hypercholesterolaemia, fibroblasts lack a surface receptor for low density lipoprotein. This results in loss of feedback inhibition on HMG CoA reductase and consequently there is great overproduction of cholesterol.

There is comparatively little information concerning the control of sterologenesis in tissues of the guinea pig, particularly the extrahepatic tissues. However it is known that hepatic sterol synthesis in the guinea pig is markedly depressed by fasting (Sauer, 1960) and by feeding either cholesterol or various bile acids (Beher et al., 1963). In a brief communication Swann and Siperstein (1972) reported that cholesterol feedback inhibition of sterologenesis in the guinea pig occurred not only in the liver but also in several extrahepatic tissues including the intestine and lung. These findings suggested that the guinea pig may be a very useful model for further studies on the effector and mechanism involved

in the feedback inhibition by cholesterol on sterologenesis. However not until recently was this work published in more detail (Swann et al., 1975). The effect of the interruption of the enterohepatic circulation of bile acids on sterol synthesis in tissues of the guinea pig apparently has not been previously determined. However Laster et al. (1966) reported that bile salts markedly inhibited sterologenesis in guinea pig intestine in vitro.

(iv) Cholesterol catabolism and excretion.

The catabolism of cholesterol to bile acids and their subsequent excretion is one of the major routes by which sterol leaves the body (Dietschy and Wilson, 1970c). Although cholesterol is also catabolised by conversion to steroid hormones, this is of negligible importance in cholesterol turnover (Chevallier, 1967). Thus this aspect of cholesterol degradation will not be discussed here.

The pathways by which cholesterol is converted to various bile acids in the liver have been described in detail by Elliot and Hyde (1971). The major pathway in bile acid synthesis first involves  $7\alpha$ -hydroxylation of cholesterol. This reaction occurs in the microsomes and is the rate-limiting step in this pathway (Danielsson et al., 1967; Shefer et al., 1970). In subsequent steps two key intermediates,  $5\beta$ -cholestane- $3\alpha,7\alpha,12\alpha$ -triol and  $5\beta$ -cholestane- $3\alpha,7\alpha$ -diol are formed. These undergo side chain cleavage in the mitochondria during which the three terminal carbons are lost. As a result of this reaction  $5\beta$ -cholestane- $3\alpha,7\alpha,12\alpha$ -triol and  $5\beta$ -cholestane- $3\alpha,7\alpha$ -diol are converted to choly CoA and chenodeoxycholy CoA respectively. In rat liver the principal bile acid

synthesised is cholic acid (Shefer et al., 1973b) whereas in the guinea pig it is chenodeoxycholic acid (Schoenfield and Sjövall, 1966). After synthesis most of the bile acid is conjugated to either glycine or taurine. Thus the four primary bile acids secreted by the liver into the bile are taurocholic acid, glycocholic acid, taurochenodeoxycholic acid and glycochenodeoxycholic acid.

After reaching the distal intestinal tract the primary bile acids may undergo bacterial transformations to a variety of deconjugated or dehydroxylated derivatives. These are the secondary bile acids and include deoxycholic acid and lithocholic acid.

Bile acids in the intestinal lumen are either excreted in the faeces or are reabsorbed. Most reabsorption occurs in the ileum and the bile acids are transported to the liver via the portal venous system. This recycling of bile acids allows them to be reused for their essential role in the absorption of fat-soluble substances from the intestinal lumen.

The enterohepatic circulation of bile acids also has a major role in the regulation of bile acid synthesis. Thus in rats biliary diversion (Myant and Eder, 1961) or cholestyramine feeding (Huff et al., 1963) results in a several-fold increase in bile acid synthesis. Although the mechanism of feedback has not been elucidated it appears to involve an effect on cholesterol 7 $\alpha$ -hydroxylase. The activity of this enzyme in rats is enhanced by biliary diversion (Danielsson et al., 1967) and cholestyramine feeding (Shefer et al., 1968; Boyd et al., 1973; Mitropoulos et al., 1973) and decreased by bile acid feeding (Shefer



et al., 1973b). The feedback inhibition may be exerted directly by bile acids or it may result from some secondary effect of bile acids such as that on cholesterol absorption. In some species bile acid synthesis is also influenced by dietary cholesterol. An increased level of cholesterol in the diet enhances bile acid excretion in rats (Wilson, 1962). It is not certain whether a similar effect occurs in humans (Wilson, 1972b).

The excretion of cholesterol and its neutral bacterial transformation products in the faeces constitutes the second major route by which sterol leaves the body (Dietschy and Wilson, 1970c). The cholesterol present in the intestinal lumen is derived from the diet and from two endogenous sources - the bile and the intestinal wall as the result of desquamation of mucosal cells. The excretion of neutral steroids is regulated largely by bile acids. This effect results because of the role of bile acids in the regulation of cholesterol absorption.

(v) Summary.

Dietary cholesterol in the intestinal lumen and cholesterol from the bile and intestinal mucosa mix and are absorbed into the intestinal wall as free cholesterol in the form of mixed micelles. This cholesterol as well as cholesterol synthesised by the intestine is esterified and incorporated into chylomicron particles which enter the intestinal lymph and reach the bloodstream via the thoracic duct. The triglyceride in chylomicra is removed in the extrahepatic tissues and the cholesterol reaches the liver contained in chylomicron remnant particles. In the liver this cholesterol is mixed with endogenous cholesterol and this pool of cholesterol can then be

(a) catabolised to bile acids, (b) excreted in the bile or (c) incorporated into lipoproteins. The bile acids are excreted in the bile and pass into the intestine where they are involved in the solubilisation of cholesterol and possibly the regulation of intestinal cholesterol synthesis. Bile acids in the intestinal lumen are either reabsorbed or excreted in the faeces.

The synthesis of cholesterol and bile acids is closely regulated by several feedback mechanisms which act to maintain a steady concentration of cholesterol in the plasma and tissues. Thus any manipulation which depletes either cholesterol or bile acids results in an enhanced synthesis of these moieties whereas expansion of the cholesterol pool results in an inhibition of cholesterol synthesis and an increase in catabolism of cholesterol to bile acids.

One aspect of cholesterol metabolism in the guinea pig which differs from that in other species is the extremely low rate of hepatic sterologogenesis. Although this has been known for a considerable time very little work has been carried out to examine the factors which regulate sterol synthesis in the liver and extrahepatic tissues of the guinea pig. The report of Swann and Siperstein (1972) that feedback inhibition of sterologogenesis by cholesterol occurred in a wide range of guinea pig tissues was significant because studies with other species, particularly the rat, had established that this feedback mechanism was restricted to the liver. The role of bile acids in the regulation of intestinal sterologogenesis has been studied in detail in rats but there have been no

definitive studies in guinea pigs. Similarly the role of the feeding pattern in the regulation of the diurnal rhythm in sterol synthesis in the liver has been studied only in rats. Thus the further study of the control of sterologogenesis in tissues of the guinea pig became the main subject of this thesis.

(b) Regulation by ascorbic acid.

(i) Cholesterol absorption and excretion.

The accumulation of cholesterol in the vitamin C-deficient animal does not appear to result from increased cholesterol absorption. Ginter et al. (1969c) found that the recovery, after 72 h, in the gut contents and faeces of 4-<sup>14</sup>C-cholesterol administered intragastrically was higher in chronically vitamin C-deficient guinea pigs than in controls. Furthermore, the recovery of radioactive cholesterol in the plasma, liver and lungs was lower in the scorbutic animals. Ascorbic acid deficiency could inhibit cholesterol absorption through its inhibition of the synthesis of bile acids (discussed below) and pancreatic lipase (Ginter et al., 1967). Decreased pancreatic lipase activity would reduce the extent of lipolysis of triglycerides to monoglycerides and fatty acids. As bile acids, monoglycerides and fatty acids are essential prerequisites for the micellar solubilisation of cholesterol (Dietschy and Wilson, 1970b) their reduced availability during vitamin C deficiency would inhibit cholesterol absorption.

As the quantification of neutral steroid output remains one of the major problems in cholesterol balance studies in guinea pigs (Ginter et al., 1973a) and in mammals in general (Dietschy and Wilson, 1970c) it is not



surprising that the effect of ascorbic acid on this aspect of cholesterol metabolism has not been studied in detail. In rabbits with advanced experimental atherosclerosis, supplementation of the diet with ascorbic acid enhances excretion of cholesterol in the bile (Novitskii, 1969). However in guinea pigs injected intraperitoneally with 4-<sup>14</sup>C-cholesterol, the output of radioactivity in the neutral steroid fraction in both bile and faeces was not different in guinea pigs with chronic latent vitamin C deficiency compared with controls (Ginter et al., 1971a). Thus present evidence would not suggest that ascorbic acid plays a major role in cholesterol excretion per se.

Some interesting effects of ascorbic acid-2-sulphate on cholesterol excretion have been reported recently. When rats were injected intracardially with <sup>35</sup>S-labelled ascorbic acid-2-sulphate, the excretion of labelled cholesteryl sulphate in the faeces was 50 times that when rats were injected with <sup>35</sup>S-labelled inorganic sulphate (Verlangieri and Mumma, 1973). Steroid sulphates usually constitute about 3% of the neutral steroid output in human faeces (Eneroth and Nyström, 1968a,b). Ascorbic acid-2-sulphate does not possess anti-scorbutic activity and does not accumulate in the liver when administered either orally or parenterally (Kuenzig et al., 1974). Obviously further work needs to be done to evaluate ascorbic acid-2-sulphate as a cholesterol-lowering agent.

(ii) Cholesterol synthesis.

The nature of the role of ascorbic acid in the regulation of cholesterol synthesis remains uncertain because the results of most studies concerning the effect

of vitamin C deficiency on sterologogenesis are conflicting. In acutely scorbutic guinea pigs the rate of sterol synthesis in various tissues has been reported to increase (Becker et al., 1953; Guchhait and Ganguli, 1961a) or to show no change (Bolker et al., 1956). In guinea pigs with chronic latent scurvy the rate of cholesterol synthesis in the liver is not affected (Ginter and Nemec, 1969). However a more recent study of baboons with latent scurvy demonstrated a significant lowering of the rate of hepatic cholesterogenesis (Weight et al., 1974).

It has been suggested that the increase in cholesterogenesis observed in acutely scorbutic guinea pigs could result from an increased availability of acetate for cholesterol synthesis (Lahiri and Banerjee, 1956; Banerjee and Singh, 1958; Guchhait and Ganguli, 1961b). Diminished oxidation of acetate occurs in liver preparations of acutely scorbutic guinea pigs. This decrease in the activity of the tricarboxylic acid cycle (Guchhait and Ganguli, 1961b) is thought to occur as a result of hypoinsulinism (Banerjee and Singh, 1958; Banerjee and Ghosh, 1960). Insulin has a role in the regulation of the activity of the tricarboxylic acid cycle (Frohman et al., 1951) and its content in the pancreas of guinea pigs with acute vitamin C deficiency is decreased (Banerjee and Ghosh, 1947). In addition, the administration of insulin to these animals increases acetylation, which is a measure of acetyl Coenzyme A availability, to normal levels and lowers the body cholesterol content to normal (Banerjee and Singh, 1958). The finding that the level of Coenzyme A (including its reduced form) does not change in severe

vitamin C deficiency was taken as further evidence that there was a genuine increase in cholesterol synthesis in the scorbutic animal. However it is difficult to accept these proposals in the light of evidence that the availability of acetate is not rate-limiting in cholesterol synthesis (Gould and Swyryd, 1966) and that hypoinsulinism may directly inhibit HMG CoA reductase (Nepokroeff et al., 1974).

Although indirect, there is evidence that ascorbic acid regulates the activity of an enzyme in the biosynthetic pathway subsequent to HMG CoA reductase; possibly squalene synthetase (Novitskii, 1971). Popják et al. (1958) showed that ascorbic acid could act as a cofactor in the conversion of labelled mevalonate to sterol by rat liver microsomes. The recent study of Weight et al. (1974) with scorbutic baboons showed that the incorporation of labelled mevalonate into cholesterol was reduced to a greater extent than was the incorporation of labelled acetate.

As discussed in a later section, bile acid synthesis is markedly reduced during vitamin C deficiency. This may in itself accelerate cholesterol synthesis indirectly by impairing the intestinal absorption of endogenous and dietary cholesterol, thereby interrupting the normal feedback inhibition on hepatic cholesterologenesis (Dietschy and Wilson, 1970a).

There is no information concerning the effect of dietary ascorbic acid deficiency on intestinal sterologogenesis. Thus as the intestine may be a major endogenous source of plasma cholesterol in humans (Cox et al., 1963) the possibility that chronic latent scurvy



influences plasma cholesterol concentration by a direct effect on the rate of cholesterol production in the intestine cannot be excluded.

(iii) Bile acid synthesis and excretion.

Several studies with guinea pigs have clearly demonstrated that bile acid synthesis and excretion are significantly reduced in vitamin C deficiency. Guchhait et al. (1963) reported that 24 h after the intraperitoneal administration of 4- $^{14}\text{C}$ -cholesterol, less radioactivity was recovered in the bile acid fraction of the liver, bile, gut and faeces of severely scorbutic guinea pigs than of pair-fed controls. Similarly Ginter et al. (1971a) found that the bile acids recovered from the liver and gallbladder bile 3 days after intraperitoneal injection of 4- $^{14}\text{C}$ -cholesterol were labelled to a lesser extent in guinea pigs with chronic latent scurvy than in the controls. When 26- $^{14}\text{C}$ -cholesterol was similarly administered, the recovery of  $^{14}\text{CO}_2$  after 10 days was less in the chronically scorbutic guinea pigs than in the control animals. Furthermore, the recovery of  $^{14}\text{CO}_2$  from the deficient animals could be significantly increased after the resaturation of the animals with ascorbic acid (Ginter et al., 1972b). Using the 26- $^{14}\text{C}$ -cholesterol technique, which slightly underestimates bile acid synthesis (Chevallier and Lutton, 1966) Ginter and his colleagues (Ginter, 1973; Ginter et al., 1973b) subsequently showed that chronically scorbutic guinea pigs produced (on a 500 g body weight basis) significantly less bile acid (8.3 mg/day) compared with control animals (11.8 mg/day). There was no difference in the rate of cholesterol turnover

between the two groups. The rate of bile acid synthesis was positively correlated with the ascorbic acid concentration of the liver which in turn was negatively correlated with the cholesterol concentration of both plasma and liver.

The mechanism by which ascorbic acid regulates bile acid synthesis is not fully understood. The conversion of cholesterol to bile acid involves a series of reactions which occur at three subcellular sites (Boyd and Percy-Robb, 1971; Elliot and Hyde, 1971). While ascorbic acid possibly regulates the oxidative steps in side chain cleavage in the mitochondria, it seems more likely to exert its major effect on the steroid nuclear hydroxylation reactions in the microsomes. Guchhait et al. (1963) showed that the formation of bile acids from 4- $^{14}\text{C}$ -cholesterol by guinea pig mitochondria was enhanced by the addition of ascorbic acid in vitro. The effect was more pronounced with mitochondria from acutely scorbutic animals than with those from the pair-fed controls. However Kritchevsky et al. (1973) were not able to demonstrate any stimulatory effect of ascorbic acid on the oxidation of 26- $^{14}\text{C}$ -cholesterol by mitochondria from normal guinea pigs or rats; probably because the mitochondrial preparations already contained the optimum concentration of ascorbic acid. Neither of these studies take into account that the 7 $\alpha$ -hydroxylation of cholesterol, which is the initial step in the primary route for the conversion of cholesterol to bile acids, occurs primarily in the microsomes. Shefer et al. (1968) have shown in studies in the rat that microsomes, on a protein weight basis,

have more than 11 times the  $7\alpha$ -hydroxylating activity than do mitochondria. Thus the role of mitochondria should be tested not with cholesterol but with steroids which have been shown to serve as mitochondrial substrates such as  $5\beta$ -cholestane- $3\alpha,7\alpha$ -diol (Boyd and Percy-Robb, 1971). An alternative pathway of bile acid synthesis has been suggested by Mitropoulos and Myant (1967a,b) in which cholesterol is first converted to 26-hydroxycholesterol. It is possible that the effect of ascorbic acid on bile acid synthesis reported by Guchhait et al. (1963) was on this pathway and not on that in which  $7\alpha$ -hydroxycholesterol is first formed.

As the basic role of ascorbic acid in metabolism is that of a hydroxylating agent (Staudinger et al., 1961; Mapson, 1967) it would seem likely that this function is extended to the regulation of bile acid synthesis. Ginter and his colleagues (Ginter et al., 1969a, 1971a, 1972b; Ginter, 1973) believe that ascorbic acid controls bile acid synthesis by regulation of the activity of the rate-limiting enzyme in the pathway, cholesterol  $7\alpha$ -hydroxylase (Danielsson et al., 1967; Shefer et al., 1970) but this is so far supported only by two lines of indirect evidence. Firstly, the concentration of cytochrome P-450 which is involved in the  $7\alpha$ -hydroxylation of cholesterol (Wada et al., 1969) is substantially reduced in scorbutic guinea pigs (Leber et al., 1970; Degkwitz et al., 1973) and this effect is reversed by repletion with ascorbic acid. Ginter and Nemec (1972) have shown that the time course of the enhancement of the oxidation of  $26\text{-}^{14}\text{C}$ -cholesterol to  $^{14}\text{CO}_2$  produced by injecting ascorbic acid into chronically



scorbutic guinea pigs closely parallels the increase in the concentration of cytochrome P-450 observed under these conditions by Leber et al. (1970). Secondly, Ginter has recently demonstrated that there is no difference in the conversion of 26- $^{14}\text{C}$ -7 $\alpha$ -hydroxycholesterol to  $^{14}\text{CO}_2$  between guinea pigs with chronic ascorbic acid deficiency and control animals (Ginter, 1975). Although this indicates that ascorbic acid regulates bile acid synthesis at the stage of 7 $\alpha$ -hydroxylation of cholesterol and not in subsequent reactions, it will only be verified by the measurement of cholesterol 7 $\alpha$ -hydroxylase in scorbutic animals.

(iv) Steroid hormone synthesis.

Although steroidogenesis constitutes a pathway of negligible importance to the overall cholesterol balance in animals (Chevallier, 1967), the involvement of ascorbic acid in its regulation has been widely studied. However many of the results are equivocal and the precise role of ascorbic acid thus remains uncertain. Most of these studies have been made on the adrenal which on stimulation by adrenocorticotrophic hormone (ACTH) releases ascorbic acid and then increases steroid hormone secretion (Slusher and Roberts, 1957; Lipscomb and Nelson, 1960).

Ascorbic acid appears to regulate two separate enzyme systems in steroidogenesis, either by a stimulatory effect on some reactions or an inhibitory effect on others. The first of these are the mitochondrial reactions which convert cholesterol to pregnenolone - these involve two successive hydroxylations to form 20 $\alpha$ ,22 $\epsilon$ -dihydroxycholesterol which, with the action of the 20,22-desmolase forms

pregnenolone. Studies with a soluble enzyme preparation from bovine and porcine adrenal mitochondria showed that while ascorbic acid was stimulatory to the  $20\alpha$ -hydroxylation reaction, it was inhibitory to 20,22-desmolase activity (Shimizu, 1970). An inhibitory effect of ascorbic acid on cholesterol side chain cleavage in rat ovary (Sulimovici and Boyd, 1968) and testis (Carballeira et al., 1974) has also been reported although high concentrations of ascorbic acid were necessary to produce such inhibitory effects.

The second series of reactions involving ascorbic acid are the mitochondrial  $11\beta$ - and  $18\beta$ -hydroxylations and the microsomal  $17\alpha$ - and 21-hydroxylations and the dehydrogenations which occur subsequently to pregnenolone formation. The  $11\beta$ -hydroxylation of deoxycortisol to cortisol is reduced in the adrenals of scorbutic guinea pigs but can be restored by the addition of ascorbic acid in vitro (Bacchus, 1957a). However when added to mitochondria from adrenals of normal guinea pigs, ascorbic acid has no effect on  $11\beta$ -hydroxylation (Spatz and Hofmann, 1965). In bovine adrenal preparations cortisol synthesis is inhibited by ascorbic acid (Hayano et al., 1956; Kitabchi, 1967) but not by dehydroascorbic acid (Hayano et al., 1956). The 21-hydroxylating activity of bovine adrenocortical microsomes is also inhibited by ascorbic acid (Cooper and Rosenthal, 1962; Kitabchi, 1965; Kitabchi et al., 1966). However as these preparations probably already contained optimum concentrations of ascorbic acid, the inhibitory effect of the added ascorbic acid may not reflect a physiological effect of ascorbic acid on 21-hydroxylase activity. Thus the activity of the various hydroxylases

involved in steroidogenesis, as with that of cholesterol 7 $\alpha$ -hydroxylase in bile acid synthesis, should be measured in scorbutic animals. There is better agreement concerning the role of ascorbic acid in the microsomal dehydrogenation reactions where studies with both guinea pig (Bacchus, 1957b) and rat (Koritz, 1963) adrenal preparations suggest a stimulatory effect on dehydrogenase activity.

(v) Summary.

The studies by Ginter and his colleagues using guinea pigs with latent scurvy have convincingly demonstrated that the most critical effect of chronic dietary inadequacy of vitamin C on cholesterol metabolism is the impairment of bile acid synthesis. Although indirect evidence strongly suggests that ascorbic acid is involved in the regulation of the rate-limiting enzyme of bile acid synthesis, cholesterol 7 $\alpha$ -hydroxylase, the effect of dietary ascorbic acid deficiency on the activity of this enzyme has not been examined. The evidence for a role of ascorbic acid in the regulation of cholesterol synthesis is conflicting. However the recent studies of Weight et al. (1974) suggest that latent scurvy results in a decreased rate of cholesterol synthesis in the liver. Their results also indicate that ascorbic acid may be involved in the regulation of an enzyme (or enzymes) subsequent to HMG CoA reductase. However this possibility has not been the subject of any further study. In addition there appears to be no information concerning the effect of dietary vitamin C deficiency on intestinal sterologenesis. Such studies with guinea pigs may have been expected because in this species the rate of intestinal sterologenesis markedly



exceeds that in the liver (Schwenk et al., 1955; Swann and Siperstein, 1972).

The study of the rate of hepatic and intestinal sterologogenesis and of the activity of cholesterol 7 $\alpha$ -hydroxylase in guinea pigs in the early stage of acute ascorbic acid deficiency has thus been the subject of some of the experiments described in this thesis. Other experiments were also carried out to further examine the effect of high levels of ascorbic acid supplementation on various aspects of cholesterol metabolism in normal guinea pigs and in guinea pigs with dietary cholesterol-induced hypercholesterolaemia.

#### MATERIALS AND METHODS

## 1. Animals.

All animals, except those used in the experiments on the effects of fasting, were derived from colonies bred at the Animal Breeding Establishment of the Australian National University. Male and female guinea pigs of an outbred albino strain were used. Unless otherwise indicated they were aged from approximately 10 to 26 weeks at the time of study. The experiments on fasting were carried out using outbred albino female guinea pigs obtained from the National Biological Standards Laboratories, Canberra, ACT. These were aged approximately 12 weeks at the time of use. Male outbred albino Wistar-derived rats, aged approximately 10 weeks at the time of study, were used. For the experiments with rabbits, females of a semi-lop randomly outbred strain aged from 12 to 24 weeks were used. Some of the animals selected for study had been used for breeding purposes.

## MATERIALS AND METHODS

### 2. Housing of animals.

The guinea pigs were weaned at from 3 to 4 weeks of age at which time they were separated according to sex and placed in wire mesh cages. They were maintained in groups of from 8 to 10 until allocated for experimental purposes. The room in which the guinea pigs were bred and maintained after weaning was freely ventilated and kept at a temperature of 20-27°C and a relative humidity of 50 ± 5% throughout the year. It was exposed to natural lighting conditions although throughout the day artificial lighting was also applied. The rabbits and rats were bred and maintained until allocation under conditions similar to those for the guinea pigs.

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After selection for experimental purposes the guinea pigs were transferred to a room from which all natural lighting had been excluded. By means of an electric timer (A. Gallenkamp & Co Ltd, London, England) the lighting was controlled such that the light and dark periods were each of 12 h length. Light was provided by 6 40W fluorescent lights from 0600 to 1800 h except during the studies on the effect of the time of lighting on feeding patterns when the normal lighting cycle was retarded by 6 h. Then the light period extended from 1200 to 2400 h. A red light of low intensity was kept glowing at all times to enable work to be undertaken during the dark period. Such light has been shown to have minimal effect on the activity of rats (McGuire et al., 1973). The temperature of this room was also maintained at 20-27°C and the relative humidity at  $50 \pm 5\%$  throughout the year. The guinea pigs were housed in wire mesh cages in groups of 3 to 6. Each cage was fitted with two glass water bottles. Unless otherwise indicated the guinea pigs were acclimatised to these conditions for at least 18 days before the commencement of experiments.

The rabbits were weaned at 5 weeks of age and the rats at 3 weeks. On transfer to the light-controlled room the rabbits were housed in single cages each of which was fitted with one feed hopper and one water bottle. An adaptation period of several weeks was allowed. Only those rats used in the feeding pattern studies and in the experiment on the diurnal rhythm in plasma cholesterol concentration were housed in the light-controlled room. The rats used for the various experiments on sterol

synthesis were housed in an adjacent room under natural lighting conditions in which the dark period lasted approximately 10 h. All rats were maintained in groups of from 3 to 5 and were housed in tins on a woodshaving litter. The tins were fitted with wire mesh tops on which the feed pellets and water bottles were placed.

### 3. Diets.

The composition and proximate analyses of the plain diets fed to guinea pigs, rabbits and rats are given in Tables 1 and 2 respectively. These diets were formulated and supplied as pellets by Bunge Aust. Pty Ltd, Murrumburrah, NSW 2595. The guinea pig and rabbit diet was also supplied in mash form. This was used for the preparation of the cholesterol and cholestyramine diets. Commercial grade cholesterol supplied by Townson and Mercer Pty Ltd, Lane Cove, NSW 2066 was used. Cholestyramine (Cuemid) was obtained from Merck, Sharp and Dohme, West Point, Pa 19486, USA. These additives were uniformly mixed with the mash in a cement mixer. The diets were then pelleted using a Provender Templewood Jr. pelleting machine fitted with a 5/32" die (Lister Blackstone Pty Ltd, Revesby, NSW 2212). The pellets were then dried in an oven at 70°C for 6-8 h. The plain diets supplied by Bunge Aust. were pelleted using a different procedure which involved compression of the mash under steam for 5 min at approximately 82°C.

The plain guinea pig and rabbit diet contained 0.023% w/w cholesterol and 0.048% w/w plant steroids. The plain diet fed to rats contained 0.023% w/w cholesterol and 0.018% w/w plant steroids. The cholesterol and cholestyramine diets used in the guinea pig studies

TABLE 1. Composition of plain diets fed to guinea pigs, rabbits and rats<sup>a</sup>.

Ingredient	Percentage composition by weight	
	Guinea Pig and Rabbit	Rat
Lucerne meal	19.93	-
Barley	4.98	14.99
Wheat	16.44	17.51
Bran	9.96	10.00
Pollard	12.18	9.18
Oats	9.96	10.00
Maize	9.96	10.00
Soyabean meal	2.77	4.17
Meat meal	6.97	13.90
Fish meal	6.60	10.00
Vitamin and mineral premixes <sup>b</sup>	0.25	0.25

<sup>a</sup>The diets were fed as pellets which in the guinea pig and rabbit diet were approximately 0.3 cm in diameter and 0.5-1.0 cm in length. Pellets in the rat diet were approximately 1.3 cm in diameter and 1.5-3.5 cm in length.

<sup>b</sup>The vitamin and mineral premixes supplemented both diets to the following amounts per kg total diet: vitamin A, 7,333 IU; vitamin D<sub>3</sub>, 1,465 IU; vitamin E, 22 IU; vitamin B<sub>1</sub>, 1.43 mg; vitamin B<sub>2</sub>, 2.86 mg; vitamin B<sub>6</sub>, 1.43 mg; vitamin B<sub>12</sub>, 7.3 µg; nicotinic acid, 22 mg; pantothenic acid, 7.3 mg; ascorbic acid, 25 mg; iron, 50.0 mg; manganese, 75.0 mg; copper, 12.5 mg; zinc, 55.0 mg; magnesium, 150.0 mg; cobalt, 1.0 mg; iodine, 1.5 mg.



TABLE 2. Analyses of plain diets fed to guinea pigs, rabbits and rats.

Component	Percentage composition by weight	
	Guinea Pig and Rabbit	Rat
Protein	18.0	22.4
Fat	3.0-3.5	4.5
Fibre	10.0	5.1
Arginine	0.98	-
Lysine (available)	-	1.11
(total)	0.97	1.31
Methionine	0.32	0.43
Methionine + cysteine	-	0.84
Cystine	0.27	-
Tryptophan	0.36	0.26
Glycine	1.13	-
Leucine	-	1.38
Isoleucine	-	0.8
Calcium	1.2	2.15
Phosphorus (available)	-	1.17
(total)	0.9	1.45
Total digestible energy kcal/g	2.26	3.04

contained, in addition to 0.048% w/w plant steroids, 0.21-0.24% w/w cholesterol and 2.5% w/w cholestyramine respectively.

While the guinea pigs were maintained in the room in which they had been bred they were fed the plain diet supplemented daily with pumpkin and either freshly cut lucerne or oats. On transfer to the light-controlled room the guinea pigs continued to receive the plain diet but this was not supplemented. Unless otherwise indicated guinea pigs in all experiments were fed the plain diet. The rabbits and rats received their respective plain diets in all experiments.

All diets were fed ad libitum except in the experiments on fasting in which the animals were fasted for 24 h. All animals had free access to water at all times. Drinking water was provided fresh daily at between 0900 and 1100 h. Feed hoppers were replenished during this time.

#### 4. Dietary vitamin C supplementation.

As described above all guinea pigs maintained in the breeding room after weaning received the plain diet which was supplemented daily with pumpkin and freshly cut fodder. Ascorbic acid was not added to their drinking water. On transfer to the light-controlled room, the guinea pigs received the same plain diet but this was not supplemented with any natural sources of vitamin C. All supplementary ascorbic acid was provided in the drinking water which was freshly prepared every day. Unless otherwise indicated, guinea pigs in all experiments were given tap water containing 0.010% w/v L-ascorbic acid

(pro analysi grade, E. Merck, 61 Darmstadt, Germany) which allowed an approximate intake of 15 mg/kg bw/day (range 14-17). In the experiments on high vitamin C intake this concentration was increased 10-, 20- or 40-fold while in the experiments on acute scurvy, the drinking water was not supplemented with ascorbic acid. Drinking water containing ascorbic acid was prepared in bulk in a glass tank and was provided in glass bottles fitted with rubber stoppers and glass nipples, thereby avoiding any contact of the ascorbic acid with metal surfaces. The diets of the rats and rabbits were not supplemented with ascorbic acid.

5. Bleeding and killing of animals, sampling of tissues and the preparation of tissue slices.

The guinea pigs were bled from an ear vein into heparinised micro blood collecting tubes (280  $\mu$ l volume, Sherwood Medical Industries, Inc, St Louis, Mo 63103, USA). These tubes were also used for the collection of blood from the tail vein of rats. At the termination of experiments animals were killed at a specific time in relation to the lighting cycle. Unless otherwise indicated this was between 3.5 and 5 h after the commencement of the dark period. Animals were killed by stunning and then exsanguinated. The blood was collected into tubes containing EDTA (final concentration 10 mM EDTA). Where appropriate, aliquots of whole blood were taken for the determination of ascorbic acid concentration. Plasma was separated from the chilled blood by centrifugation. Organs were quickly excised and placed in ice-cold saline (0.9% w/v). Their weights were recorded before samples of tissue were removed for the preparation of tissue slices or extraction of lipids and



ascorbic acid. In the experiments with guinea pigs the gallbladder was removed from the liver after its excision. Sections of the gastrointestinal tract were freed of mesentery, opened longitudinally and washed thoroughly in ice-cold saline. Skin was obtained from the backs of guinea pigs after the hair had been removed. For the experiments on sterol synthesis tissue slices were prepared freehand with a razor blade to a thickness of approximately 0.8-1.0 mm. Slices of liver tissue were consistently prepared from the left lobe, the largest lobe of the liver. The adrenals were not sliced. Tissues and plasma not used for immediate analysis were stored at  $-20^{\circ}\text{C}$ .

#### 6. Chemicals.

1- $^{14}\text{C}$ -acetate, 2- $^{14}\text{C}$ -mevalonic acid lactone and 4- $^{14}\text{C}$ -cholesterol were obtained from The Radiochemical Centre, Amersham, Bucks, UK. The 4- $^{14}\text{C}$ -cholesterol was purified by thin-layer chromatography immediately before use. DL-mevalonic acid lactone (DL- $\beta$ -hydroxy- $\beta$ -methyl- $\delta$ -valeric lactone), NADPH (nicotinamide adenine dinucleotide phosphate, reduced form),  $\beta$ -mercaptoethylamine hydrochloride (cysteamine HCl), cholesterol (5-cholesten-3 $\beta$ -ol) and 5 $\alpha$ -cholestane were supplied by Sigma Chemical Co, St Louis, Mo 63118, USA. Reference samples of 7 $\alpha$ -hydroxycholesterol (5-cholesten-3 $\beta$ , 7 $\alpha$ -diol) and 7-ketocholesterol (5-cholesten-3 $\beta$ -ol-7-one) were obtained from Steraloids Inc, Pawling, NY 12564, USA. Sodium acetate, glycerol trioleate, 2,4-dinitrophenylhydrazine and activated charcoal were supplied by British Drug Houses Ltd, Poole, UK. Aluminium oxide (neutral) and

silica gel G were supplied by E. Merck, 61 Darmstadt, Germany. Digitonin was obtained from both BDH and Merck. Florisil was supplied by Floridin Co, Hancock, W Va, USA. Tween 80 (polyoxyethylene(20)sorbitan mono-oleate) was obtained from Atlas Chemical Industries Inc, Wilmington, Del 19899, USA. 2,5-diphenyloxazole (PPO) was supplied by Koch-Light Labs Ltd, Colnbrook, Bucks, UK and Ultraphor by BASF Australia Ltd, Sydney, NSW 2017. O-phthaldialdehyde (puriss grade) was obtained from Fluka AG, Bucks SG, Switzerland. All other reagents were commercial analytical grade products. Petroleum ether (b.p. 60-80°C) and all other solvents, except toluene, were redistilled before use.

## 7. Chemical methods.

### (a) Cholesterol in plasma.

The saponification and extraction procedure described by Mann (1961) was applied directly to samples of plasma. Cholesterol in the petroleum ether extract was measured using the o-phthaldialdehyde colour reagent described by Zlatkis and Zak (1969).

### (b) Cholesterol and cholesteryl ester in plasma and tissues.

Plasma and tissue samples were extracted and the lipid extracts washed by the method of Folch et al. (1957). The free cholesterol and cholesteryl ester were separated by chromatographing samples of the extract on thin layers (0.5 mm thick) of silica gel G coated on glass plates (20 cm × 20 cm) with petroleum ether-diethyl ether-glacial acetic acid (70:30:2 by volume) as solvent. Reference samples of cholesterol and cholesteryl stearate were run on each plate. The lipid bands were visualised under u.v.

light after spraying with Ultraphor (0.01% w/v in water). The cholesterol and cholesteryl ester bands were each scraped into sintered glass funnels (porosity 3) and eluted with petroleum ether-diethyl ether (1:1 v/v, 30 ml). The cholesteryl ester was hydrolysed and extracted as described above. The unesterified cholesterol and the cholesterol derived from the cholesteryl ester were assayed as described above.

(c) Cholesterol and plant steroids in feed.

The proportions of cholesterol and plant steroids in the plain diets were determined by a combination of thin-layer and gas-liquid chromatographic techniques. 5 g of diet was heated under reflux with 130 ml 0.77 N HCl in 77% v/v aqueous ethanol for 4 h. After cooling 10 N KOH (20 ml) and water (20 ml) were added and the mixture extracted with petroleum ether (3 × 50 ml). An aliquot of the combined extracts was separated on thin-layer chromatographic plates coated with florisil as described by Miettinen et al. (1965). The band corresponding to the cholesterol and plant steroids was scraped off and eluted with petroleum ether-diethyl ether (1:1 v/v, 30 ml). The proportions of cholesterol and plant steroids were then determined using gas-liquid chromatography with 5 $\alpha$ -cholestane as the internal standard. The gas-liquid chromatographic (GLC) analyses were carried out by Dr C. E. West.

The cholesterol content of the diet to which cholesterol had been added was determined by a different procedure. 5 g of diet was heated under reflux in 130 ml 0.77 N KOH in 77% v/v aqueous ethanol for 2 h. After



cooling, water (40 ml) was added and the mixture extracted with petroleum ether (50 ml). After suitable dilution the cholesterol concentration of the petroleum ether phase was measured as described above. As o-phthaldialdehyde also reacts with plant steroids, the calculation of the cholesterol content of the diet required a correction for the plant steroid content which was determined by GLC.

(d) Triglyceride in plasma.

The plasma triglyceride concentration was measured as described by Neri and Frings (1973) except that 0.5 ml samples of plasma were used. Glycerol trioleate was used as the standard.

(e) Ascorbic acid in blood and tissues.

Ascorbic acid was assayed by the method of Schaffert and Kingsley (1955). The proteins in blood were precipitated by adding trichloroacetic acid (36% w/v, 2 ml) to 8 ml blood. Tissues were homogenised in trichloroacetic acid (final concentration 4% w/v). The extracts were centrifuged and the resultant supernatants treated with activated charcoal to oxidise the ascorbic acid and remove the pigment. The dehydroascorbic acid was measured by coupling to 2,4-dinitrophenylhydrazine.

(f) Preparation of 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol.

7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol were prepared via 7 $\alpha$ -formoxycholesteryl benzoate. 7 $\alpha$ -bromocholesteryl benzoate, prepared as described by Bide et al. (1948), was treated with sodium formate by the method of Henbest and Jones (1948). This yielded 7 $\alpha$ -formoxycholesteryl benzoate which was then saponified. The saponification mixture was extracted with diethyl ether and the extract

then washed with water to remove the alkali. The ether was evaporated and the residue dissolved in ethanol. Samples of this were separated on thin layers of silica gel G (0.5 mm thick) on glass plates (20 cm × 20 cm) with benzene-ethyl acetate (3:7 v/v) as solvent. As shown in Figure 1 two compounds of  $R_{f(\text{rel})}$  0.45 and 0.56 appeared below that of cholesterol ( $R_{f(\text{rel})}$  of cholesterol = 1). The former corresponded to that of the reference  $7\alpha$ -hydroxycholesterol. Further confirmation of the identification of this compound as  $7\alpha$ -hydroxycholesterol and identification of the other compound as  $7\beta$ -hydroxycholesterol were obtained by comparing the chromatographic separation with that described by Van Cantfort (1972). Larger amounts of each compound were purified using the same chromatographic procedure except that the samples were applied across the full width of each plate.

Dr T. G. Redgrave provided additional  $7\alpha$ -hydroxycholesterol prepared by a modification of the method of Starka (1961). Chloroform was used as solvent instead of acetic acid and the reaction was carried out at room temperature. The resultant diacetate was purified by column chromatography and after saponification, the  $7\alpha$ -hydroxycholesterol was separated by TLC.

(g) Assay of radioactivity.

A liquid scintillation counter (Model LS100, Beckman Instruments Inc, Fullerton, Calif 92634, USA) was used for all assays of radioactivity.

8. Assay of sterol and fatty acid synthesis.

The assay of sterol synthesis was carried out



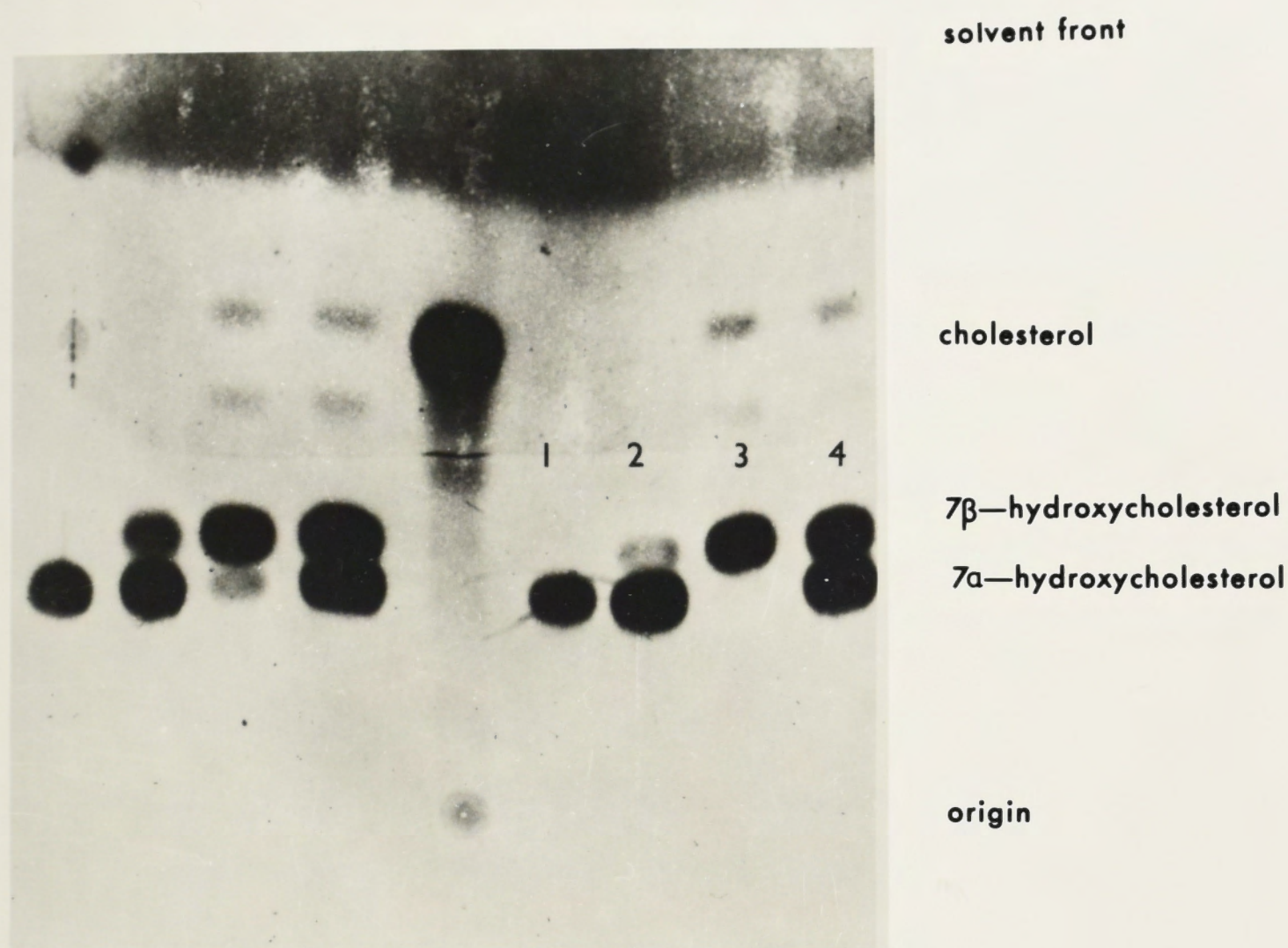


Figure 1. *Thin-layer chromatographic separation of cholesterol and its 7 $\alpha$ - and 7 $\beta$ -hydroxylated derivatives.* Reference samples of cholesterol and 7 $\alpha$ -hydroxycholesterol and the 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol prepared via 7 $\alpha$ -formoxycholesteryl benzoate, were separated at room temperature on a thin layer of silica gel G (0.5 mm thick) on a glass plate (20 cm x 20 cm) with benzene-ethyl acetate (3:7 v/v) as solvent. The sterols were visualised by spraying with sulphuric acid (50% v/v) and heating at 110°C for 1 h. 1, reference 7 $\alpha$ -hydroxycholesterol; 2 and 3, 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol respectively (prepared by the method described); 4, the separation obtained when samples of 2 and 3 were applied together. The unlabelled set of spots on the left correspond to those designated as 1-4 above but from a preceding preparation in which the degree of purity of the products obtained was lower.



essentially as described by Sabine et al. (1967) except that only in the initial experiments on diurnal variations in liver sterol synthesis was Krebs-Ringer bicarbonate buffer used. This was used at pH 7.4 and contained acetate at a concentration of 2 mM. In all subsequent experiments Krebs-Ringer phosphate buffer was used. Both buffers were prepared as described by Cohen (1957). The preparation of the phosphate buffer was more convenient than that for the bicarbonate buffer. Tissue slices (200 mg) were incubated in triplicate in 2 ml buffer containing 1-<sup>14</sup>C-acetate (100 $\mu$ Ci/mmol) or 2-<sup>14</sup>C-mevalonate (50 $\mu$ Ci/mmol). In the experiments with guinea pigs the liver was incubated at pH 6.5 and the lung at pH 6.0, both in buffer containing 10 mM acetate while the ileum and all other tissues were incubated at pH 7.5 in buffer containing 5 mM acetate. In the studies in which 2-<sup>14</sup>C-mevalonate was used as the substrate the liver and ileum were incubated in buffer containing 1 mM and 5 mM mevalonate respectively. Rat liver and ileum were incubated at pH 7.4 in the presence of 10 mM acetate. Incubations in bicarbonate buffer were carried out in an atmosphere of 95% oxygen/5% carbon dioxide while those in phosphate buffer were carried out in 100% oxygen. All incubations were carried out at 37°C for 2 h. During the incubation the flasks were shaken in a reciprocating water bath (Paton Industries, Pty Ltd, Stepney, SA 5069) at a rate of 100 oscillations/min. Incubations were stopped by the addition of 0.2N sulphuric acid (1 ml). Blanks were prepared by the addition of acid to the medium before incubation. After centrifugation of the incubation mixture the supernatant was discarded and

the tissue slices saponified in 2.5 ml of 2N KOH in ethanol (80% v/v). Saponifications were carried out at 70°C for 12 h. After cooling, 1 ml water was added and the mixture extracted with petroleum ether (2 × 6 ml). After evaporation of the solvent under nitrogen, the sterol was dissolved in ethanol-acetone (1:1 v/v, 4 ml) and then digitonin (0.5% w/v in ethanol-water 1:1 v/v, 2 ml) and 2 drops of acetic acid (10% v/v) were added. After standing for 12 h with intermittent mixing, the mixtures were centrifuged and the digitonide precipitates were washed with acetone-diethyl ether (1:2 v/v, 6 ml) followed by 6 ml diethyl ether and then dissolved in 1 ml methanol. When fatty acid synthesis was also measured, the saponification mixture was acidified and extracted with hexane (2 × 5 ml). The hexane was evaporated and the residues dissolved in 1 ml methanol. The radioactivity of the samples in methanol was measured after the addition of 2,5-diphenyloxazole in toluene (0.5% w/v, 10 ml). Counting errors due to quenching were negligible.

In the initial experiments on diurnal variations in liver sterol synthesis, the incorporation of  $1\text{-}^{14}\text{C}$ -acetate into total digitonin-precipitable sterols was expressed as a percentage of the total activity added to the incubation. In all subsequent experiments sterol synthesis was expressed as  $\mu\text{moles } 1\text{-}^{14}\text{C}$ -acetate or  $2\text{-}^{14}\text{C}$ -mevalonate incorporated into total digitonin-precipitable sterols/g wet tissue/h. The rate of incorporation of  $1\text{-}^{14}\text{C}$ -acetate into fatty acids was also expressed on this basis.

9. Assay of cholesterol 7 $\alpha$ -hydroxylase.

Cholesterol 7 $\alpha$ -hydroxylase was assayed by a modification of the method developed for rat liver preparations by Mitton et al. (1971). All operations after excision of the liver were carried out at 0-4°C. 5 g of liver was finely minced with scissors and homogenised in 20 ml of potassium phosphate buffer (0.1 M. pH 7.4) containing EDTA (1 mM). The tissue was homogenised in 3 strokes using a Potter-Elvehjem teflon-glass homogeniser with a loose-fitting pestle. The clearance between the pestle and tube was approximately 0.4 mm. The homogenate was then centrifuged at 18,000xg for 15 min and the resultant supernatant, termed S<sub>18</sub>, was used immediately for the assay.

The incubation mixture (2.2 ml) contained 1.5 ml S<sub>18</sub>, potassium phosphate buffer (0.1M, pH 7.4),  $\beta$ -mercaptoethylamine (15 mM), MgCl<sub>2</sub> (4 mM) and NADPH (1 mM). The cholesterol substrate was prepared by mixing 33  $\mu$ g of 4-<sup>14</sup>C-cholesterol (58.7 mCi/mmol) with 400  $\mu$ l of Tween 80 in acetone (50 mg/ml). The acetone was evaporated and 180  $\mu$ l water added. The preparation was mixed thoroughly. 10  $\mu$ l of this preparation, containing approximately 0.25 $\mu$ Ci 4-<sup>14</sup>C-cholesterol (1.65  $\mu$ g) was added to each incubation.

Incubations were carried out in air at 37°C for 30 min during which time the flasks were shaken at 100 oscillations/min. The incubations and all subsequent procedures were performed in semi-darkness. For each sample of S<sub>18</sub>, three incubations were carried out. Two of these contained active enzyme preparation while the



third contained inactive  $S_{18}$ . The enzyme was inactivated by heating the sample over a flame until all the protein had precipitated. The third incubation thus provided a measure of the autoxidation of the 4- $^{14}\text{C}$ -cholesterol during the incubation. For each series of assays a zero-time blank was also prepared by adding methanol (13 ml) to the incubation mixture immediately after addition of the 4- $^{14}\text{C}$ -cholesterol. This provided a measure of the level of autoxidation which resulted during the ensuing analytical procedures.

After the incubation had been stopped by the addition of methanol (13 ml), chloroform (26 ml) was added. Samples were allowed to stand for 6 h at 4°C in darkness, after which time the precipitated protein was removed by centrifugation. The lipid-containing supernatant was washed by the method of Folch et al. (1957) and the supernatant evaporated under nitrogen. The extracts, dissolved in chloroform-methanol (2:1 v/v), were co-chromatographed with reference 7 $\alpha$ -hydroxycholesterol on thin layers of silica gel G (0.5 mm thick) on glass plates (20 cm wide  $\times$  30 cm high) at 4°C using diethyl ether as solvent. Reference samples of cholesterol, 7-ketocholesterol, 7 $\alpha$ -hydroxycholesterol and 7 $\beta$ -hydroxycholesterol were also run on each chromatographic plate. After spraying with Ultraphor (0.01% w/v in water), the separated bands and reference samples were visualised under u.v. light. The separation obtained is shown in Figure 2. The bands corresponding to 7 $\alpha$ -hydroxycholesterol, 7 $\beta$ -hydroxycholesterol, 7-ketocholesterol and cholesterol as well as the origin and the band intermediate between the origin and



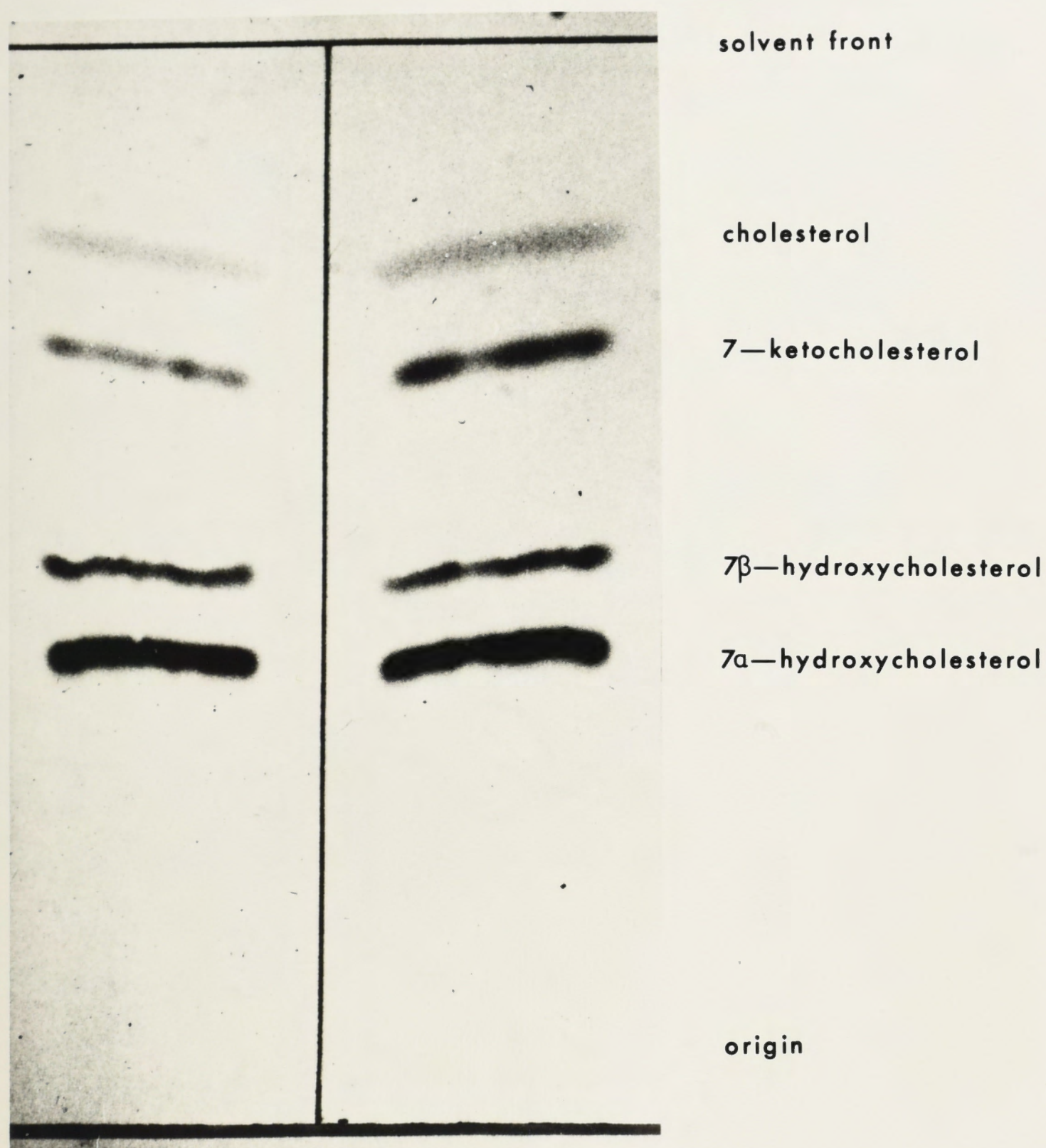


Figure 2. Thin-layer chromatographic separation of the reaction products formed during the assay of cholesterol 7 $\alpha$ -hydroxylase. Reference samples of cholesterol, 7-ketocholesterol, and 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol were chromatographed on a thin layer of silica gel G (0.5 mm thick) on a glass plate (20 cm wide x 30 cm high) using diethyl ether as solvent at 4°C. The sterols were visualised by spraying with sulphuric acid (50% v/v) and heating at 110°C for 1 h.



7 $\alpha$ -hydroxycholesterol, were scraped directly into counting vials. 10 ml of toluene containing 2,5-diphenyloxazole (0.5% w/v) was added and the samples assayed for radioactivity. No correction for quenching due to the presence of the gel in the scintillator was necessary (Mitton et al., 1971). The radioactivity in each band was expressed as a percentage of the total radioactivity recovered from all six bands.

10. Studies on diurnal variations in cholesterol metabolism in guinea pigs and in plasma cholesterol concentration in rats.

In the first experiment with guinea pigs 21 males were killed in groups of 3 at 4-hourly intervals. The first group was killed at 0600 h which was the commencement of the light period. Liver sterol synthesis and the concentrations of total cholesterol in the plasma, liver and adrenal were measured. The proportions of free and esterified cholesterol in the plasma, liver and adrenal were also determined. In the second experiment liver sterol synthesis was measured in male guinea pigs killed at four time points. Four animals were killed at 0900 h and 5 animals at each of 1100, 2100 and 2300 h.

In the study with rats, two experiments were carried out and the data combined. In each experiment a group of 3 males was bled from the tail vein at 4-hourly intervals commencing at 0600 h. Different groups of animals were used at each time point. The plasma total cholesterol concentration was determined.



11. Studies on feeding patterns of guinea pigs, rabbits and rats.

In these studies feed intakes were determined by calculating the difference in the total weight of the container and feed at consecutive time points 1 h apart. Feed containers were readily detachable from the cages and could thus be removed for weighing with minimal disturbance to the animals. Spillage of feed was negligible. Each series of measurements was completed within 10 min and the animals remained undisturbed at all other times apart from when fresh drinking water was supplied. Water intake was measured in the same way as feed intake.

(a) Guinea pigs.

Two experiments on guinea pigs were carried out. In the first the normal feeding pattern of males and females was studied. There were four groups of males, each containing 5 animals and two groups of females each containing 3 animals. Hourly measurements of feed intake were made for 29 h commencing at 0800 h, 2 h after the commencement of the light period. The second experiment was designed to study the effect of the lighting cycle on the feeding pattern. In this experiment, 7 males and 7 females, housed as two groups of 3 and 4 were studied. The hourly feed consumptions of these animals were recorded on two occasions (days -12 and -4) while they were maintained under a lighting regime in which the light period extended from 0600 to 1800 h. On day 0 the lighting cycle was retarded by 6 h such that the light period then extended from 1200 to 2400 h. The feeding pattern of the 4 groups of guinea pigs was again studied 8 and 15 days

after this phase shift. On all four occasions the hourly measurements of feed intake commenced at 0600 h and continued for 24 h.

(b) Rabbits.

The feeding pattern of rabbits was studied in a series of three experiments in all of which individually caged females were used. In the first experiment the hourly feed intakes of 11 animals were recorded for 24 h commencing at 0900 h. In the second experiment the feed consumption of 12 animals was measured over 2 consecutive days. At the same time water intake was also measured. The third experiment was identical in design to the second experiment described for the guinea pigs and was carried out at the same time as the guinea pig study. Nine rabbits were used in the third experiment.

(c) Rats.

The normal feeding pattern of rats was studied by measuring the hourly feed intakes of 5 groups of males, each containing 4 animals, for 24 h commencing at 0900 h.

12. Studies on the optimal conditions for the assay in vitro of sterologogenesis in guinea pig and rat tissues.

The effect of pH on the incorporation of  $1\text{-}^{14}\text{C}$ -acetate into total digitonin-precipitable sterols by slices of guinea pig liver, ileum and lung was studied using the tissues derived from 3 males. The pH range used for each tissue was liver, 5.5-7.5; ileum, 6.0-8.0 and lung, 5.0-7.0. The appropriate range for each tissue was established in preliminary experiments. The optimum pH for sterol synthesis in rat liver and ileum was not determined.

The effect of acetate concentration on the rate of sterol synthesis in the liver, ileum and lung of the guinea pig was investigated by varying the acetate concentration of the incubation medium over the range 0.2 to 20 mM while simultaneously maintaining a constant specific activity of the substrate. For each tissue a separate group of 4 animals was used. A similar experiment was carried out using rat liver and ileum. Two male rats were killed at mid-night and their respective tissues combined.

The linearity of the incorporation of  $1\text{-}^{14}\text{C}$ -acetate into sterol over a 2-h incubation period was demonstrated using guinea pig liver, ileum and lung. Incubations were carried out for 1 and 2 h using tissues derived from 4 males.

The effect of mevalonate concentration in the incubation medium on sterol synthesis in guinea pig liver and ileum was studied in a series of three experiments in each of which the liver and ileum from 1 male were used. A different range of concentration of mevalonate was used in each experiment and overall this extended from 0.6 to 9.2 mM.

### 13. Studies on the relative rates of sterologenesis in guinea pig and rat tissues.

The relative rates of sterol synthesis in guinea pig tissues were studied in a series of four experiments. In all experiments, except the fourth, sterol synthesis was measured using  $1\text{-}^{14}\text{C}$ -acetate. In the fourth experiment both  $1\text{-}^{14}\text{C}$ -acetate and  $2\text{-}^{14}\text{C}$ -mevalonate were used.

In the first experiment groups of male guinea pigs were killed at 6-hourly intervals over an 18-h period



commencing at 2100 h, 3 h after the onset of the dark period. Sterol synthesis was measured in the liver, ileum and lung. At both 2100 and 0900 h, 10 animals were used while at 0300 and 1500 h there were 7 animals. This involved two separate studies and the data for each were combined.

In the second experiment the relative rates of sterol synthesis in the liver and various regions of the gastrointestinal tract in both male and female guinea pigs were examined. A total of 4 males and 6 females was used.

The third experiment examined the rates of sterol synthesis in the skin, thigh muscle, epididymal adipose tissue, adrenal and testis of male guinea pigs. For each tissue only 2 or 3 animals were used. The tissues were not all derived from the same animals.

The fourth experiment examined the effect of age on sterol synthesis in the liver and ileum. Tissues were derived from 2 groups of male guinea pigs. In the younger group there were 4 animals aged 7 weeks while the older group contained 3 animals aged 29 weeks. Sterol synthesis was measured from both  $1\text{-}^{14}\text{C}$ -acetate and  $2\text{-}^{14}\text{C}$ -mevalonate.

The study on the relative rates of hepatic and intestinal sterologogenesis in rats was carried out on males maintained under a natural lighting cycle. Five animals were killed at mid-night and 5 at mid-day and the incorporation of  $1\text{-}^{14}\text{C}$ -acetate into total digitonin-precipitable sterols by the liver and ileum measured.

#### 14. Studies on the effects of cholesterol and cholestyramine feeding and of fasting on sterologogenesis in guinea pig tissues.

The effects of feeding cholesterol and cholestyramine and of fasting on sterologogenesis in guinea pig tissues were

studied in a series of four experiments.

In the first experiment 3 groups of female guinea pigs each containing 6 animals were fed the plain diet or a diet containing added cholesterol or cholestyramine. Plasma cholesterol concentrations were determined before the commencement of feeding the diets. Initial and final body weights were recorded and feed consumption was also measured towards the end of the experiment. The animals fed the cholestyramine, plain and cholesterol diets were killed on days 14, 15 and 16 of the experiment respectively. All animals were killed approximately 4 h after the commencement of the dark period. Sterol synthesis from  $1\text{-}^{14}\text{C}$ -acetate was measured in the liver, ileum and lung. The plasma and tissue cholesterol concentrations were also measured.

The second experiment was similar to the first except that males were used and fatty acid synthesis in the liver, ileum and lung was also measured. There were 4 animals in each group. The animals fed the cholestyramine, plain and cholesterol diets were killed on days 13, 14 and 15 of the experiment respectively.

In the third experiment the effect of cholestyramine feeding on hepatic and intestinal sterol synthesis was further studied. Two groups of male guinea pigs each containing 4 animals were used. One group received the plain diet and the other the cholestyramine diet. The cholestyramine- and plain-fed animals were killed on days 14 and 15 of the experiment respectively. Both groups were killed approximately 4 h after the commencement of the light period. Sterol synthesis from both  $1\text{-}^{14}\text{C}$ -acetate

and 2-<sup>14</sup>C-mevalonate in the liver and ileum was measured as were the plasma and tissue cholesterol concentrations.

In the fourth experiment the effect of fasting on sterol synthesis was examined. Two studies were carried out and the data combined. Two groups of females were used. The first group containing 8 animals continued to be fed ad libitum while the second group containing 9 animals was fasted for 24 h. Body weights were recorded 24 h before and at the time of killing. All animals were killed about 4 h after the commencement of the dark period and both sterol and fatty acid synthesis from 1-<sup>14</sup>C-acetate in the liver, ileum and lung were measured. The plasma and tissue cholesterol concentrations were also measured.

15. Studies on the effects of severe ascorbic acid deficiency on cholesterol metabolism in guinea pigs.

All guinea pigs used in this study were adapted to conditions in the light-controlled room for only 4 days before the commencement of the experiments. In the experiment with the unsupplemented animals, 5 males and 3 females were fed the plain diet for 23 days without ascorbic acid supplementation. Plasma cholesterol concentration was measured on days 0, 10 and 17. Body weights were recorded at 3-day intervals up until day 12. After day 14, body weights were recorded daily until day 23. Feed consumption was measured at 3-day intervals. On days 0, 19 and 23 all animals were individually photographed. On day 23 all animals were killed and the concentrations of cholesterol in plasma, liver and adrenal and of ascorbic acid in liver and adrenal measured.



In the experiment with the supplemented animals there were 5 males and 5 females. These animals received 0.010% w/v ascorbic acid in their drinking water. As these groups of animals were the controls for another series of experiments being carried out at that time, the days on which the animals were weighed and their plasma cholesterol determined, varied from those described for the unsupplemented animals. Body weights were recorded and plasma cholesterol concentrations determined at weekly intervals. Feed consumption was recorded at 3-day intervals. As these animals were not killed until a much longer period after the completion of the experiment with the unsupplemented animals, the values for cholesterol and ascorbic acid concentrations in normal guinea pigs of similar age were taken from other experiments.

16. Studies on the effect of acute ascorbic acid deficiency on sterologenesis in the liver and ileum of guinea pigs.

Nine male guinea pigs were divided into two groups containing 4 and 5 animals respectively, such that the mean plasma cholesterol concentration of both was similar. Both groups were fed the plain diet throughout the experiment. The group containing 4 animals received 0.010% w/v ascorbic acid in their drinking water while the group containing 5 animals did not receive any ascorbic acid supplementation. Body weights were recorded at the commencement of the experiment and when the animals were killed. The unsupplemented and supplemented animals were killed on days 15 and 16 of the experiment respectively. All animals were killed approximately 4 h after the commencement of the dark period. Sterol synthesis from

1-<sup>14</sup>C-acetate and 2-<sup>14</sup>C-mevalonate in the liver and ileum was measured. The concentrations of cholesterol in the plasma, liver, ileum and adrenal and of ascorbic acid in whole blood, liver, ileum and adrenal were also measured.

At about the same time as the above experiment was carried out, 4 male guinea pigs were taken directly from the room in which they had been bred, and killed. These animals were the same age as those used in the experiment on acute scurvy and sterol synthesis but were fed the plain diet supplemented with pumpkin and freshly cut fodder. The concentrations of cholesterol in the plasma, liver and adrenal and of ascorbic acid in whole blood, liver and adrenal of these animals were measured.

17. Studies on the effects of acute ascorbic acid deficiency and cholestyramine feeding on cholesterol 7 $\alpha$ -hydroxylase in guinea pigs.

Twelve male guinea pigs were divided into groups of 4 each having similar mean plasma cholesterol concentrations. Two groups received the plain diet while the third group was fed the diet containing cholestyramine. One of the groups fed the plain diet did not receive ascorbic acid supplementation while the other two groups were given normal levels of ascorbic acid supplementation. Initial and final body weights were recorded. The animals fed the cholestyramine diet and those fed the plain diet without ascorbic acid supplementation were killed on days 14 and 15 of the experiment respectively. Two of the animals fed the plain diet with ascorbic acid supplementation were also killed on each of these days. All animals were killed approximately 6 h after the commencement of the

dark period and the activity of cholesterol 7 $\alpha$ -hydroxylase measured. The concentrations of cholesterol in the plasma and liver and of ascorbic acid in whole blood and liver were also measured.

18. Studies on the effects of high levels of ascorbic acid supplementation on cholesterol metabolism in guinea pigs.

Three experiments were carried out. In the first, the effect of a prolonged high level of ascorbic acid supplementation on cholesterol metabolism in female guinea pigs was examined. The plasma cholesterol concentrations of 10 female guinea pigs maintained in the room where they had been bred were determined and the animals then immediately transferred to the light-controlled room. The animals were placed in two groups each having similar mean plasma cholesterol concentrations. Both groups were fed the plain diet. The first group received 0.010% w/v ascorbic acid in their drinking water while the second group was given 10 times this concentration. For the first 4 weeks the plasma cholesterol concentration of all animals was determined at weekly intervals. Animals were then maintained for a further 28 weeks on their respective levels of ascorbic acid supplementation. The body weights of all animals were recorded weekly. In the 27th week, one of the animals in the group receiving the control level of supplementation, developed an acute infection and had to be eliminated from the experiment. The remaining 9 animals were killed at the end of the 32nd week. Sterol synthesis from both 1-<sup>14</sup>C-acetate and 2-<sup>14</sup>C-mevalonate in the liver and ileum was measured. The concentrations of cholesterol in the plasma, liver, ileum and adrenal and of ascorbic



acid in whole blood, liver, ileum and adrenal were also measured. Plasma triglyceride concentration was also determined.

About 2 months after the completion of this study, another study was carried out in which the plasma cholesterol concentrations of 10 female guinea pigs were determined at weekly intervals over a 4-week period while they were maintained in the room where they had been bred. These guinea pigs were fed the plain diet and received natural sources of ascorbic acid supplementation.

In the second experiment the effect of high levels of ascorbic acid supplementation on cholesterol metabolism in female guinea pigs fed the plain and cholesterol diets was examined. One group, containing 4 animals received the plain diet while another group containing 5 animals was given a diet to which cholesterol had been added. Each group received its respective diet throughout the experiment. The plasma cholesterol concentration of both groups was measured on day 0 and again on day 22. During this time both groups received 0.010% w/v ascorbic acid in their drinking water. From day 22 to day 30 this concentration was increased 10-fold. Plasma cholesterol concentrations were determined on day 30. The concentration of ascorbic acid in the drinking water was then increased to 0.200% w/v and both groups of animals received this level of supplementation until day 51. Plasma cholesterol concentration was determined on days 37, 43 and 51. From day 51 until day 75 the level of supplementation was reduced to 0.010% w/v. Plasma cholesterol concentration was determined on day 62. All animals were weighed at the

same time as they were bled. On day 75 all animals were killed and the concentrations of cholesterol in the plasma, liver and adrenal and of ascorbic acid in whole blood, liver and adrenal were measured.

The third experiment examined the effect of high levels of ascorbic acid supplementation on cholesterol metabolism in male guinea pigs. Two groups, each containing 5 animals, received the plain diet throughout the experiment. Both groups received 0.010% w/v ascorbic acid in their drinking water for the first week. For the following two weeks this concentration was increased 10-fold in one group and 20-fold in the other group. In the 4th and 5th weeks the first group continued to receive 0.100% w/v ascorbic acid in their drinking water while in the second group the concentration was increased to 0.400% w/v. Plasma cholesterol concentration was measured in all animals at the beginning of the experiment and at weekly intervals thereafter. Body weights were recorded at the time the animals were bled. At the end of the 5th week all animals were killed and the concentrations of cholesterol in plasma and of ascorbic acid in whole blood and liver were measured.

## PART I. DIURNAL VARIATIONS IN CHOLESTEROL METABOLISM

While several studies with rats have clearly demonstrated that the synthesis of cholesterol (Back *et al.*, 1969; Horton *et al.*, 1970; Edwards *et al.*, 1972) and its conversion to bile acids (Chevallier and Lutton, 1966; Danielsson, 1972; Gislen *et al.*, 1973) exhibit a marked diurnal variation, the extent to which the plasma and tissue cholesterol concentrations also show such variation had not been clearly established. Furthermore, there did not appear to be any information concerning the nature and extent of diurnal variations in any aspect of cholesterol metabolism in guinea pigs. The present series of experiments sought to provide such information which could then be used in planning subsequent studies.

### RESULTS AND DISCUSSION

#### (a) Results

The first two experiments involved a study of the diurnal variability of hepatic sterologensis and of plasma and tissue cholesterol concentrations in the guinea pig. As shown in Figure 3 there was a marked diurnal variation in liver sterol synthesis with maximum activity at 2200 h, 4 h after the commencement of the dark period. However the variation between animals at this time point was very high and the average rate of sterologensis at 2200 h did not differ significantly from that observed at any other time point. Thus a second experiment was carried out in which a much larger number of animals was studied at about the times when liver sterol synthesis occurred at minimal and maximal rates in the first experiment. As shown in Table 3, hepatic sterologensis



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TABLE 3. Liver sterol synthesis in male guinea pigs at various times throughout a 24-h period.

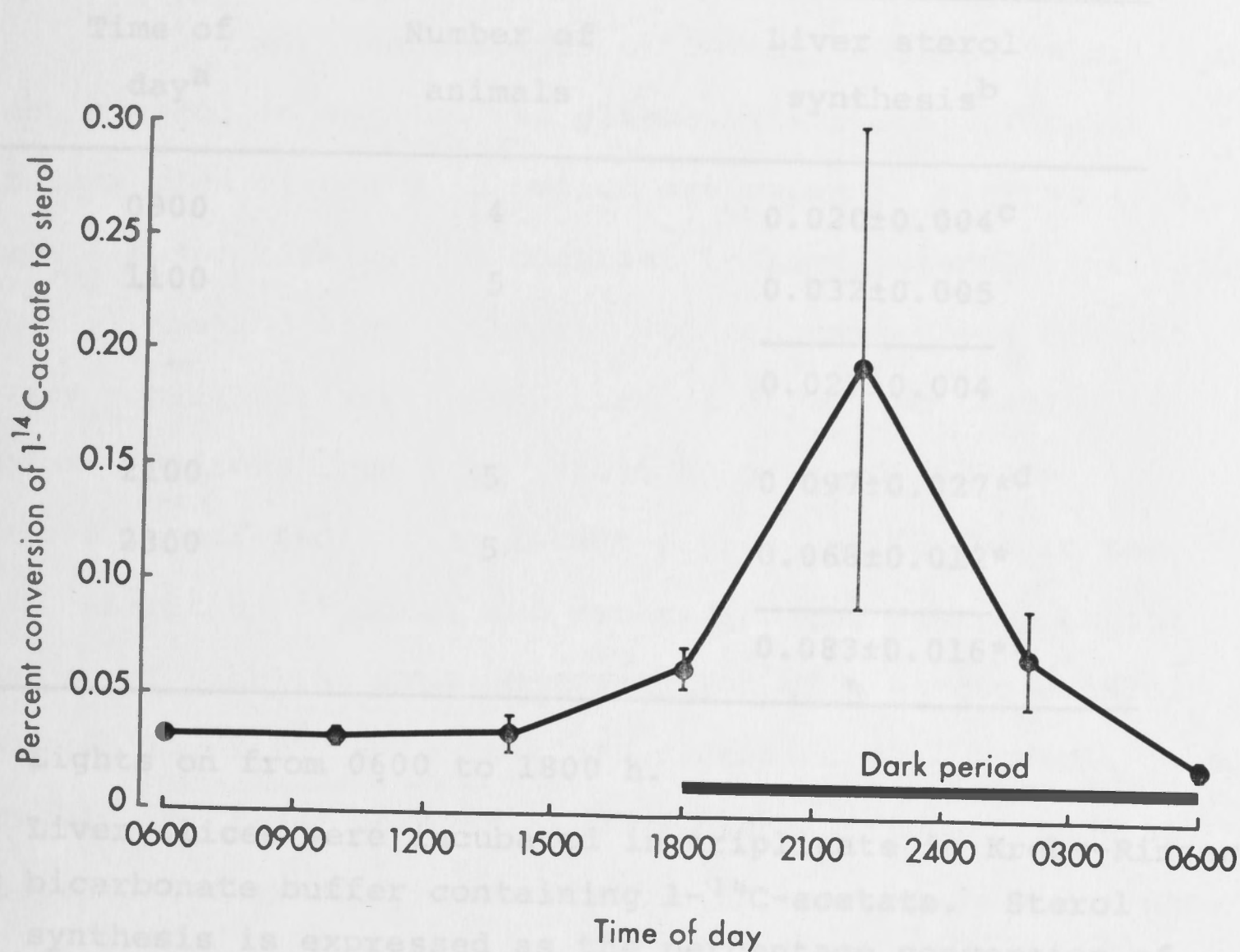


Figure 3. Diurnal variation in liver sterol synthesis in male guinea pigs. Animals were killed at 4-hourly intervals and the incorporation of  $1-^{14}\text{C}$ -acetate into total digitonin-precipitable sterols by liver slices measured. Incubations were carried out in triplicate in Krebs-Ringer bicarbonate buffer. Values are the mean  $\pm$  S.E. of determinations from 3 animals.

TABLE 3. Liver sterol synthesis in male guinea pigs at various times throughout a 24-h period.

Time of day <sup>a</sup>	Number of animals	Liver sterol synthesis <sup>b</sup>
0900	4	0.020±0.004 <sup>c</sup>
1100	5	0.032±0.005
		0.027±0.004
2100	5	0.097±0.027* <sup>d</sup>
2300	5	0.068±0.012*
		0.083±0.016**

a Lights on from 0600 to 1800 h.

b Liver slices were incubated in triplicate in Krebs-Ringer bicarbonate buffer containing 1-<sup>14</sup>C-acetate. Sterol synthesis is expressed as the percentage conversion of 1-<sup>14</sup>C-acetate to total digitonin-precipitable sterols.

c Values are the mean±S.E.

d Comparison by Student's t-test with value 12 h earlier:

\* P < 0.05

\*\* P < 0.01

was significantly higher (P < 0.05) 3 and 5 h after the commencement of the dark period than at the respective time points 12 h earlier. The diurnal rhythm of hepatic sterol synthesis in the guinea pig was thus qualitatively similar to that in rats fed ad libitum and maintained under controlled lighting (Horton et al., 1970; Edwards et al., 1972). However the maximum rate of synthesis in the guinea pig was only about 4% of that reported in the



rat (Horton et al., 1970) using the same assay. This difference had also been demonstrated in vivo by Beher et al. (1963).

The concentrations of total, free and esterified cholesterol in each of the plasma, liver and adrenal of guinea pigs over a 24-h period are shown in Figures 4, 5 and 6 respectively. In contrast to liver sterol synthesis, the plasma and tissue cholesterol concentrations did not vary consistently with the time of day. The result for plasma differs from that reported by Laird and Fox (1970) who demonstrated a significant diurnal variation in the concentration of total and esterified cholesterol in the serum of rabbits. The concentration of free cholesterol varied inversely with that of esterified cholesterol. The result for liver however is consistent with reports that hepatic cholesterol concentration in the rat does not show a diurnal variation (Bortz and Steele, 1973; Nervi et al., 1975).

As the first study with guinea pigs involved measurements on only 3 animals at each time point, it was possible that the variation in plasma cholesterol concentration between animals may have exceeded any diurnal fluctuation. Thus in the study with rats a larger number of animals was used. The plasma cholesterol concentration was determined in groups of 6 animals at 4-hourly intervals. As shown in Figure 7 the plasma cholesterol concentration did not vary significantly over the 24-h period, thus confirming the findings with the guinea pigs. In normocholesterolaemic humans, plasma cholesterol concentration does not show a diurnal variation (Shapiro et al., 1959).

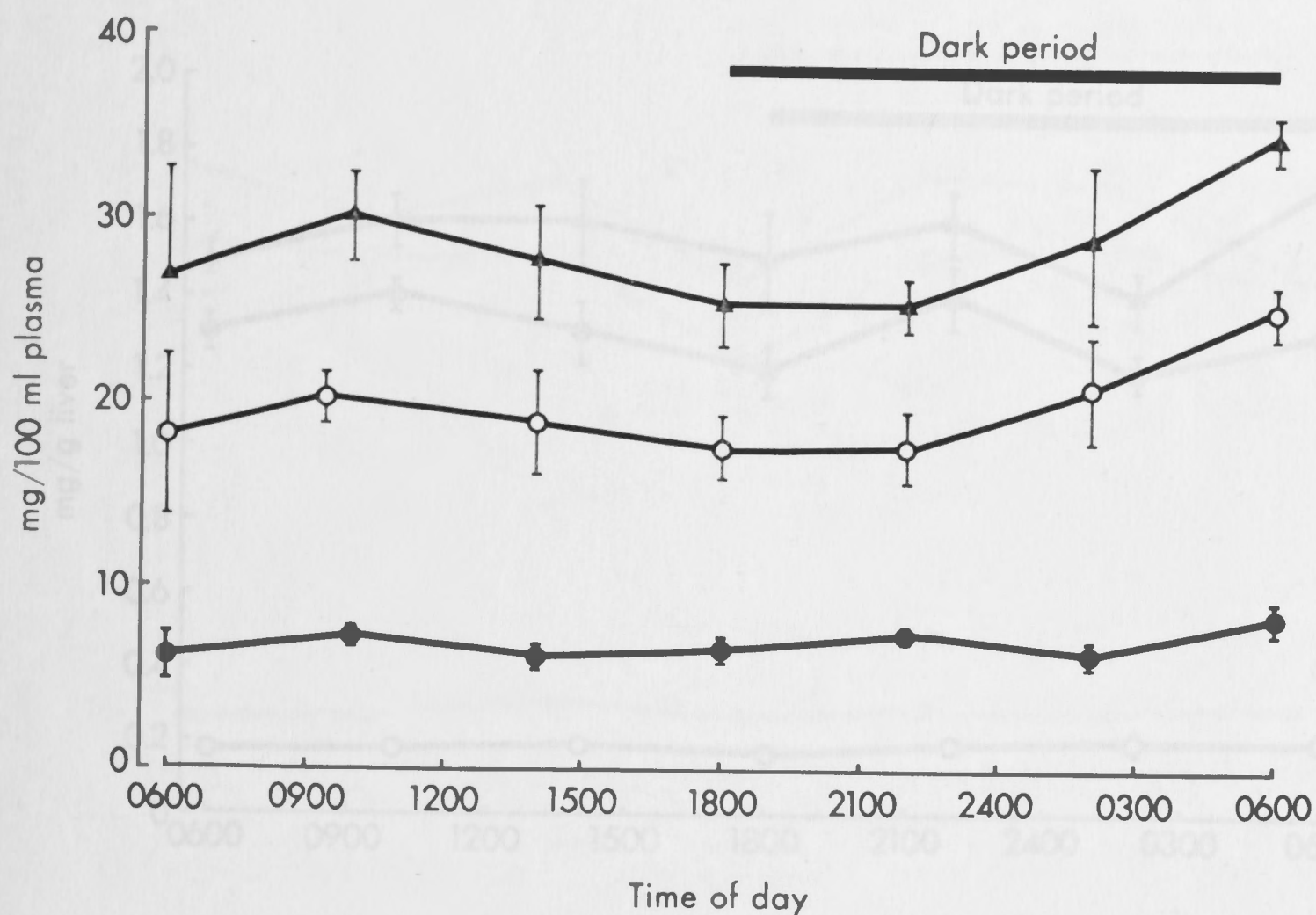


Figure 4. Plasma cholesterol concentration in male guinea pigs over a 24-h period. Groups of animals were killed at 4-hourly intervals and the concentrations of total (▲—▲), free (●—●) and esterified (○—○) cholesterol in plasma measured. Each value is the mean±S.E. of determinations from 3 animals.

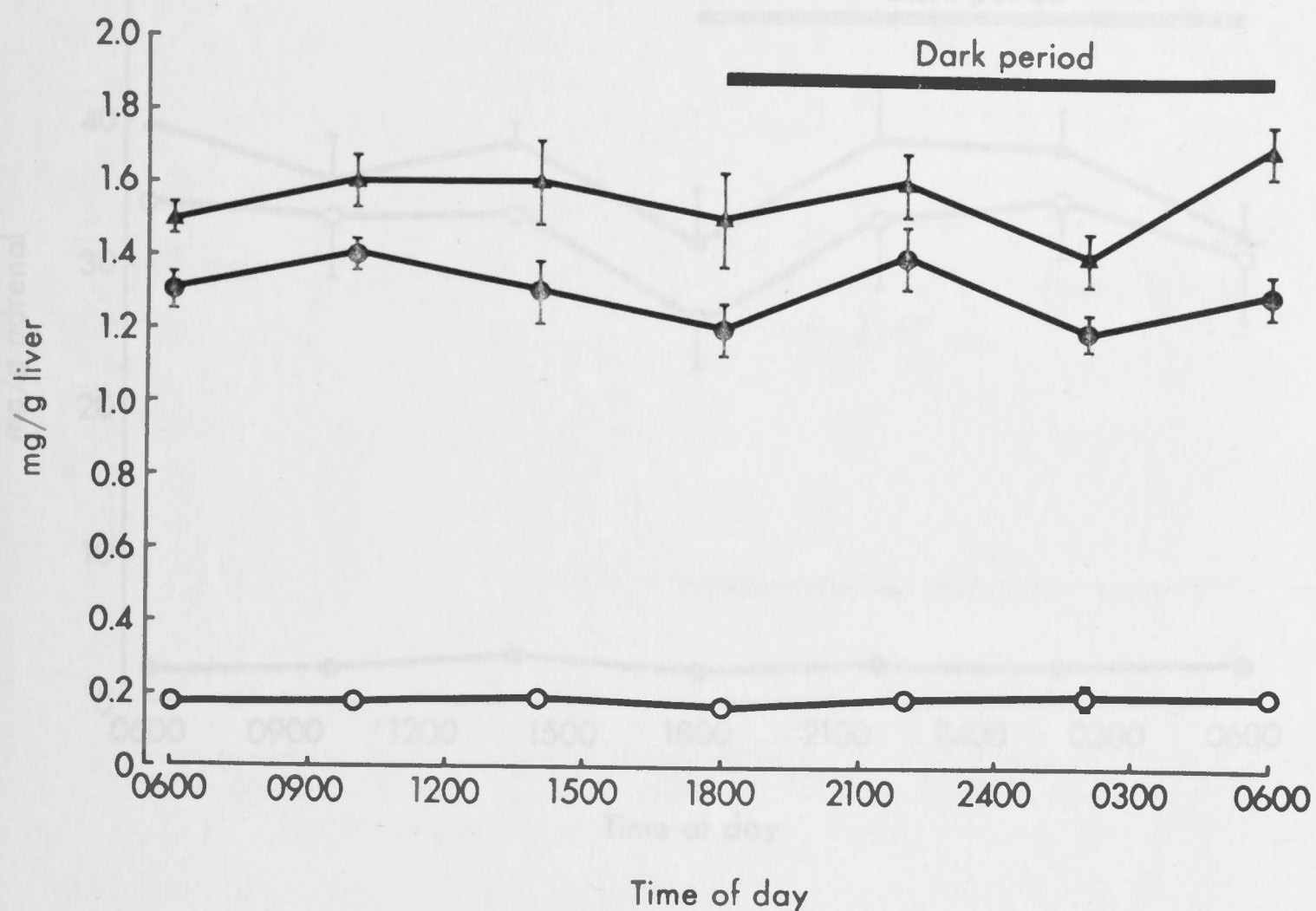


Figure 5. Liver cholesterol concentration in male guinea pigs over a 24-h period. Groups of animals were killed at 4-hourly intervals and the concentrations of total ( $\blacktriangle$ — $\blacktriangle$ ), free ( $\bullet$ — $\bullet$ ) and esterified ( $\circ$ — $\circ$ ) cholesterol in liver measured. Values are expressed on a wet weight basis and are the mean  $\pm$  S.E. of determinations from 3 animals.



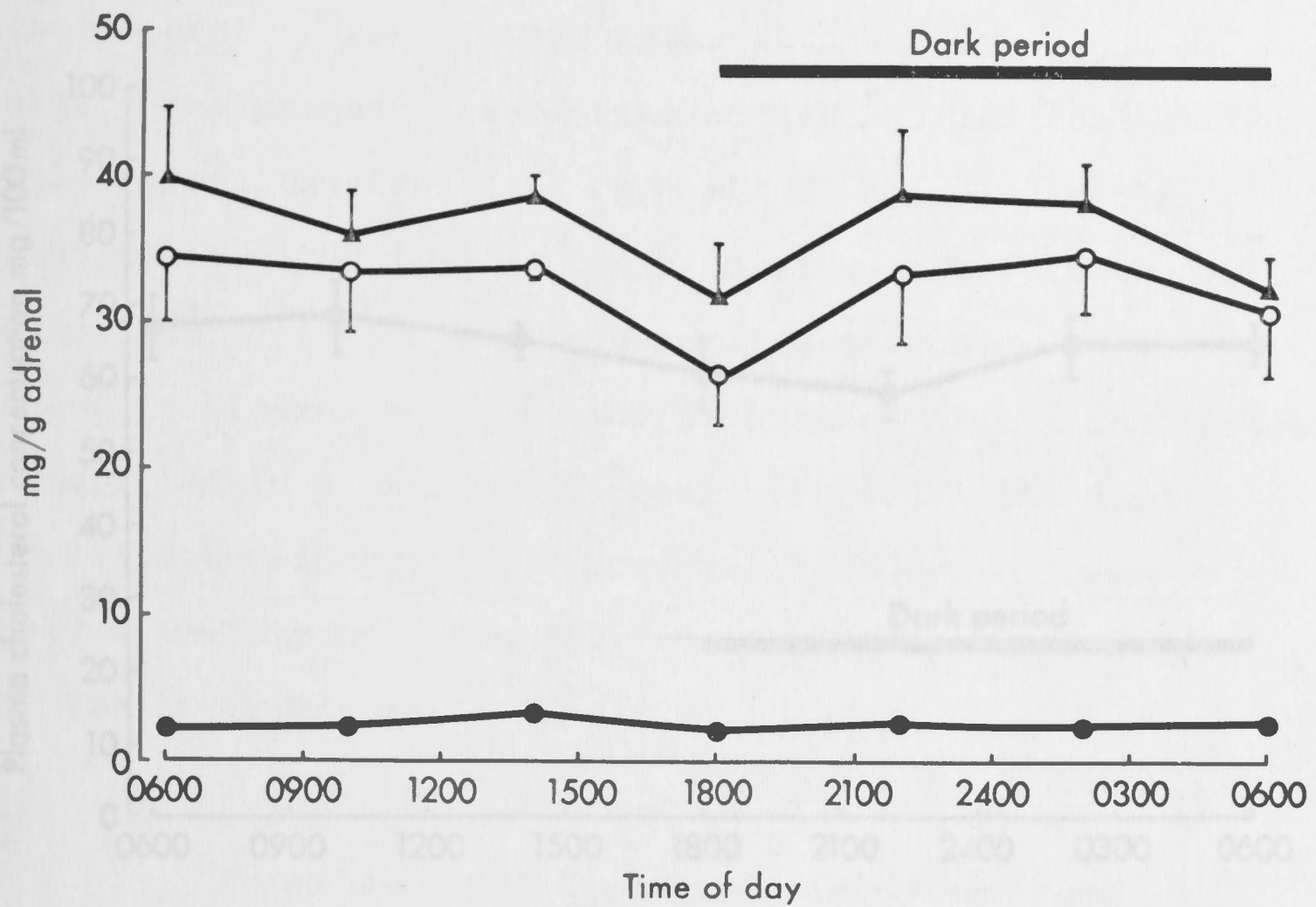


Figure 6. Adrenal cholesterol concentration in male guinea pigs over a 24-h period. Groups of animals were killed at 4-hourly intervals and the concentrations of total ( $\blacktriangle$ — $\blacktriangle$ ), free ( $\bullet$ — $\bullet$ ) and esterified ( $\circ$ — $\circ$ ) cholesterol in adrenals measured. Values are expressed on a wet weight basis and are the mean  $\pm$  S.E. of determinations from 3 animals.

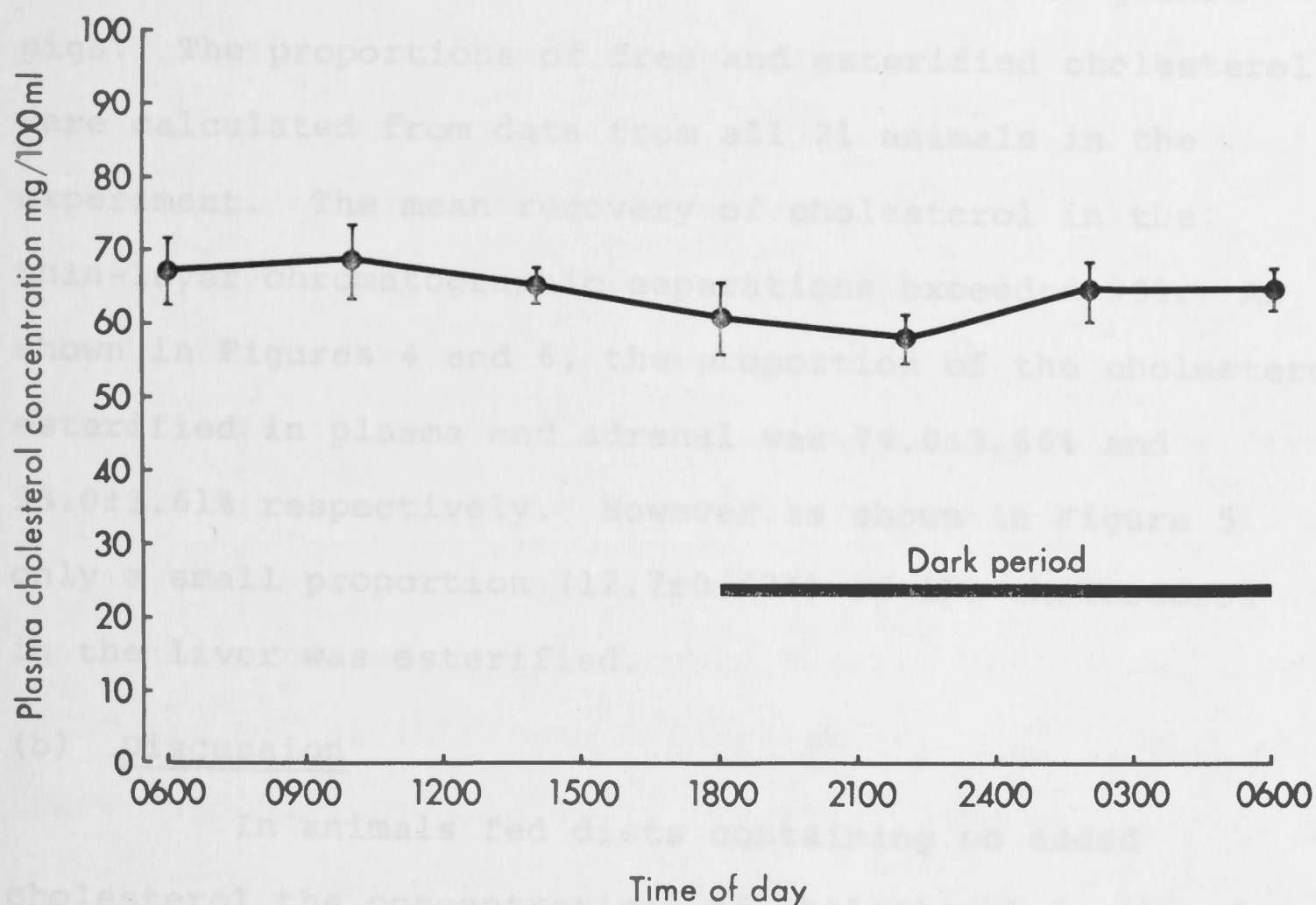


Figure 7. Plasma cholesterol concentration in male rats over a 24-h period. Animals were bled from the tail vein at 4-hourly intervals and the plasma total cholesterol concentration measured. A different group of animals was used at each time point. Values are the mean  $\pm$  S.E. of determinations from 6 animals.

In addition to establishing the lack of any significant diurnal fluctuations in cholesterol concentrations, the first experiment also provided information on the proportions of free and esterified cholesterol in the plasma, liver and adrenal of guinea pigs. The proportions of free and esterified cholesterol were calculated from data from all 21 animals in the experiment. The mean recovery of cholesterol in the thin-layer chromatographic separations exceeded 95%. As shown in Figures 4 and 6, the proportion of the cholesterol esterified in plasma and adrenal was  $74.0 \pm 3.66\%$  and  $93.0 \pm 3.61\%$  respectively. However as shown in Figure 5 only a small proportion ( $12.7 \pm 0.42\%$ ) of the cholesterol in the liver was esterified.

#### (b) Discussion

In animals fed diets containing no added cholesterol the concentrations of cholesterol in the plasma and tissues would be expected to be constant throughout the 24-h period because of the close balance between the rates of biosynthesis of cholesterol and bile acids. Although Danielsson (1972) showed that changes in the rates of bile acid and cholesterol synthesis occur almost concomitantly, Takeuchi et al. (1974), in agreement with Myant and Eder (1961), demonstrated that changes in cholesterol synthesis precede changes in bile acid synthesis. The work of Takeuchi et al. (1974) provided evidence that increased hepatic cholesterol synthesis enhances cholesterol 7 $\alpha$ -hydroxylase activity.

The effect of feeding a diet containing a high cholesterol content on the diurnal stability of plasma



cholesterol concentration differs between species. Rand and Quackenbush (1965) demonstrated a diurnal variation in the plasma cholesterol concentration of rats in which hypercholesterolaemia had been produced by feeding cholesterol in the diet. This variation was associated with the feeding period rather than with the light-dark cycle. In humans however, the intake of a large single dose of cholesterol with a meal, does not affect the diurnal stability of plasma cholesterol concentration (Turner and Steiner, 1939). This difference between species probably reflects the greater capacity of the rat intestine to absorb cholesterol compared with that of the human (Dietschy and Wilson, 1970b).

In rats the inhibition of hepatic cholesterol synthesis produced by cholesterol feeding (Gould and Swyryd, 1966) and by the intravenous administration of lipoproteins (Nervi and Dietschy, 1975) is invariably associated with an elevation in the cholesteryl ester content of the liver. However this does not necessarily imply that cholesteryl ester is the feedback effector (Nervi and Dietschy, 1975). In contrast, the diurnal variation in cholesterol synthesis in rat liver is not associated with any change in the content of cholesteryl esters in the hepatocyte (Nervi et al., 1975). The present studies with guinea pigs confirmed this observation. Despite a marked increase in hepatic sterologogenesis during the dark period, the concentration of cholesteryl ester in the liver remained constant throughout the 24-h period. The correlation coefficient obtained by comparing the rate of hepatic sterologogenesis with hepatic cholesteryl ester concentration in all 21 animals was  $-0.07$ . The identity of the effector (or effectors)

responsible for the diurnal variation in hepatic cholesterol synthesis has not been clearly established although evidence suggests that several hormones are involved (Dugan et al., 1974; Huber et al., 1974; Nepokroeff et al., 1974). Studies with rats have shown that the timing of the diurnal rhythm in liver cholesterol synthesis is regulated by the cyclic pattern of feed intake (Dugan et al., 1972; Edwards et al., 1972). This possibly occurs by a direct influence of feeding on the relative concentrations of those hormones which regulate the rate of cholesterol synthesis (Dugan et al., 1974). Unfortunately the relationship between the diurnal variation in feed intake and cholesterol synthesis has not been studied in other species. As there appeared to be no information on the feeding pattern of guinea pigs this was studied in a series of experiments, the results of which are described in Part II.

#### (a) Results.

The first series of experiments involved a comparative study of the normal pattern of feed intake in guinea pigs, rabbits and rats. In the first experiment with guinea pigs hourly measurements of feed intake were made for 24 h commencing at 0800 h, 2 h after the commencement of the light period. The observed feeding pattern for the 24-h period extending from 1200 h is shown in Figure 3. Both the males and

## PART II. FEEDING PATTERNS OF THE GUINEA PIG, RABBIT AND RAT

Most studies on the relationship between the diurnal rhythm of cholesterol synthesis and the pattern of feed intake have been carried out using rats which consume most of their feed during the dark period. The diurnal rhythms of hepatic and intestinal cholesterologenesis in rats fed ad libitum reach a maximum at about the middle of the dark period (Edwards et al., 1972). While the results presented in Part I demonstrated that the diurnal rhythm of hepatic sterologogenesis in guinea pigs was similar to that reported in rats, preliminary measurements showed that the feed intake of guinea pigs in the light period was not different from that in the dark period, suggesting that the feeding pattern of guinea pigs was different from that shown by rats. The present series of experiments were carried out to determine whether guinea pigs showed a diurnal rhythm in feed intake and to examine the role of the lighting cycle in the control of such a rhythm. Comparative studies on the feeding patterns of rabbits and rats were also carried out.

### (a) Results.

The first series of experiments involved a comparative study of the normal pattern of feed intake in guinea pigs, rabbits and rats. In the first experiment with guinea pigs hourly measurements of feed intake were made for 29 h commencing at 0800 h, 2 h after the commencement of the light period. The observed feeding pattern for the 24-h period extending from 1200 h is shown in Figure 8. Both the males and



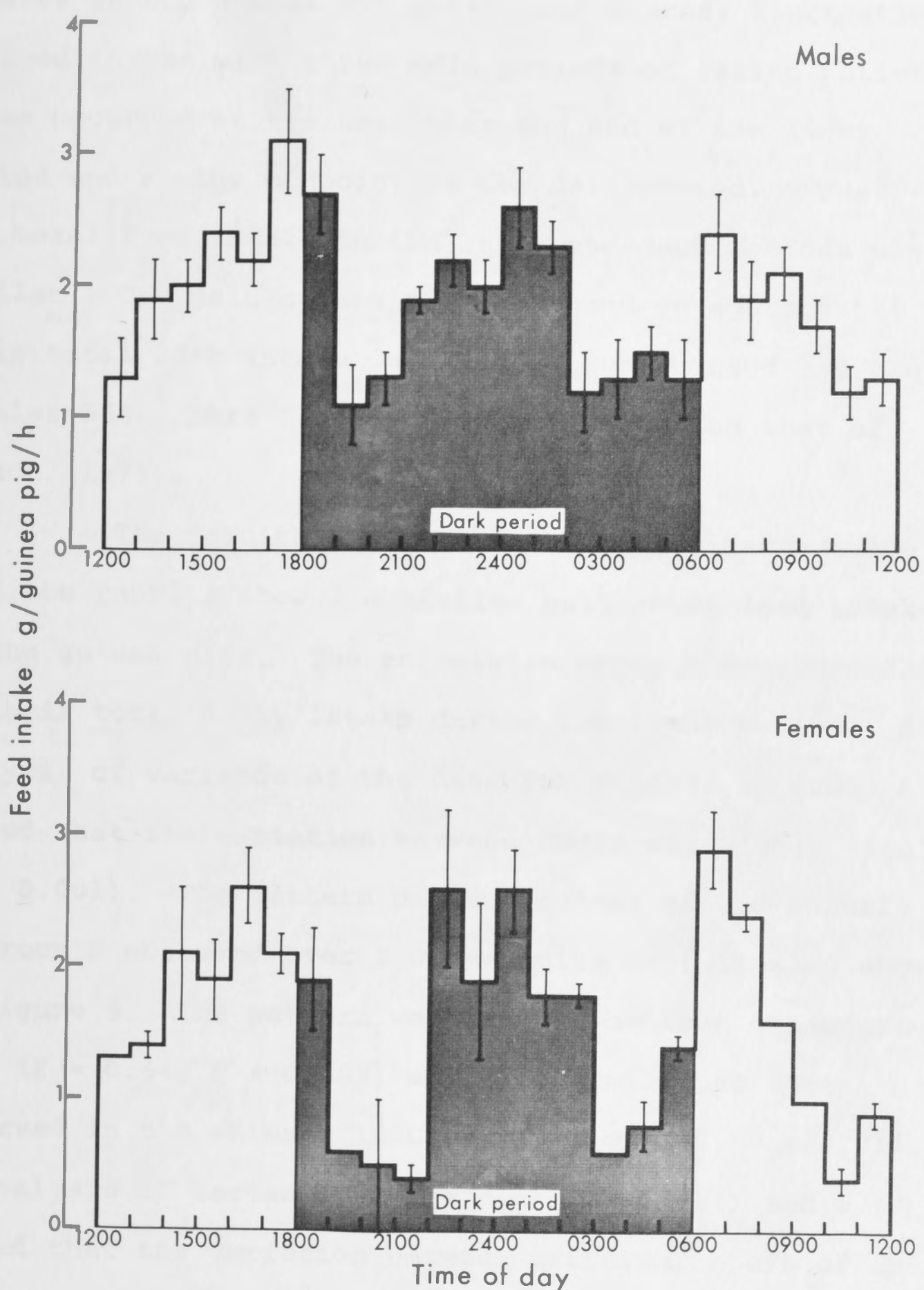


Figure 8. Diurnal variation in the feed intake of male and female guinea pigs. The hourly feed intakes of 4 groups of males, each containing 5 animals and of 2 groups of females, each containing 3 animals, were measured for 29 h. The light period was from 0600 to 1800 h. Values are the mean $\pm$ S.E.

females showed a weak but consistent diurnal fluctuation in feed intake with three main periods of eating activity. These occurred at the beginning and end of the light period and at the midpoint of the dark period. However, the total feed intake in the light and dark periods was similar. The male guinea pigs consumed on average 52% of their total 24-h intake during the light period and the females 55%. This finding is consistent with that of Hirsch (1973).

The results described in Figure 9 demonstrate that the rabbits showed a similar pattern of feed intake to the guinea pigs. The animals in Group A consumed 53% of their total daily intake during the light period. An analysis of variance of the data for animals in Group A showed that the variation between hours was highly significant ( $P < 0.001$ ). The pattern of feed intake of the animals in Group B observed over 2 consecutive days is also shown in Figure 9. The pattern was similar between consecutive days ( $r = 0.64$ ,  $P < 0.002$ ) and also similar to that observed in the animals in Group A ( $r = 0.52$ ,  $P < 0.01$ ). An analysis of variance of the data for days 1 and 2 showed that the variation between different hours of the day was highly significant ( $P < 0.001$ ) confirming that the rabbits ate significantly different amounts at different times of the day. There was also a highly significant variation ( $P < 0.001$ ) between the rabbits, as individual rabbits differed markedly in their overall feed intake. There was no significant variation between days, rabbits-days, or hours-days, confirming that the rabbits were consistent in their eating patterns from day

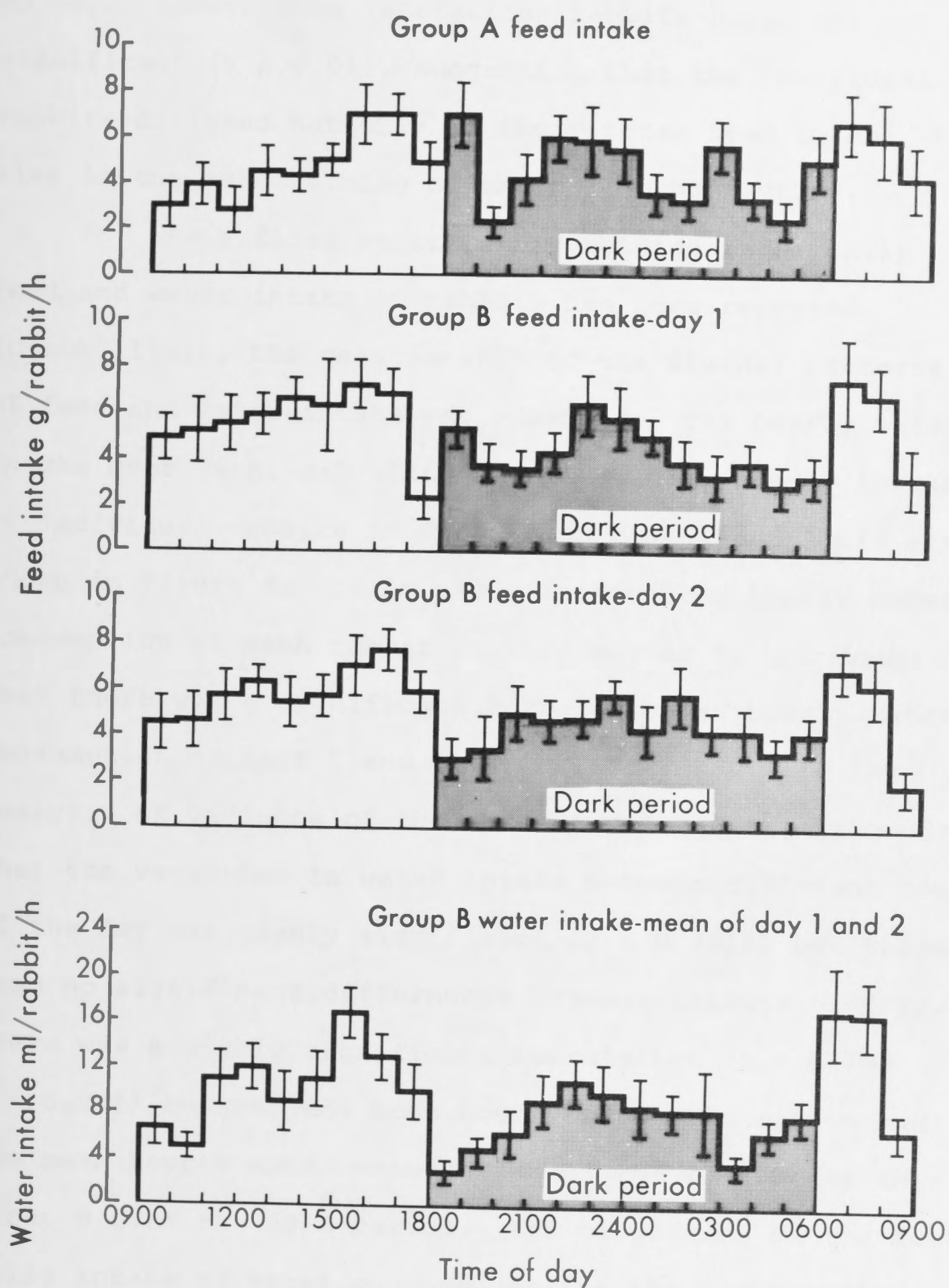


Figure 9. Diurnal variation in the feed and water intake of female rabbits. For Group A in which there were 11 animals, hourly feed intakes were measured for 24 h. For Group B in which there were 12 animals, the hourly feed and water intakes were measured for 48 h. The light period was from 0600 to 1800 h. Values are the mean  $\pm$  S.E.



to day. However the interaction rabbits-hours was significant ( $P < 0.01$ ), suggesting that the individual rabbits differed not only in their total feed intake, but also in the exact timing of their feeding rhythms.

As a fixed relationship between total daily feed and water intake of rabbits has been reported (Cizek, 1961), the relationship of the diurnal patterns of feed and water intake was examined. The hourly water intake over 24 h, calculated from the mean hourly intake of individual rabbits in Group B on days 1 and 2, is also shown in Figure 9. It is valid to pool the hourly water consumption of each rabbit in this way as it was found that there was a significant correlation of hourly water consumption on days 1 and 2 ( $r = 0.59$ ;  $P < 0.01$ ). An analysis of variance of the data on water intake showed that the variation in water intake between different hours of the day was highly significant ( $P < 0.001$ ), but there were no significant differences between rabbits or days. There was a highly significant correlation ( $R = 0.73$ ;  $P < 0.001$ ) between the mean hourly feed consumption and the mean hourly water consumption of the 12 rabbits in Group B over the 48-h period. The proportion of the total daily intake of water consumed during the light period was 60% and during the corresponding period the proportion of feed consumed was 57%.

The diurnal rhythm of feed intake in guinea pigs and rabbits differed markedly from that shown by rats (Figure 10). Unlike the guinea pigs and rabbits, the rats consumed most of their total 24-h intake during the dark period. Eating activity during the light period was

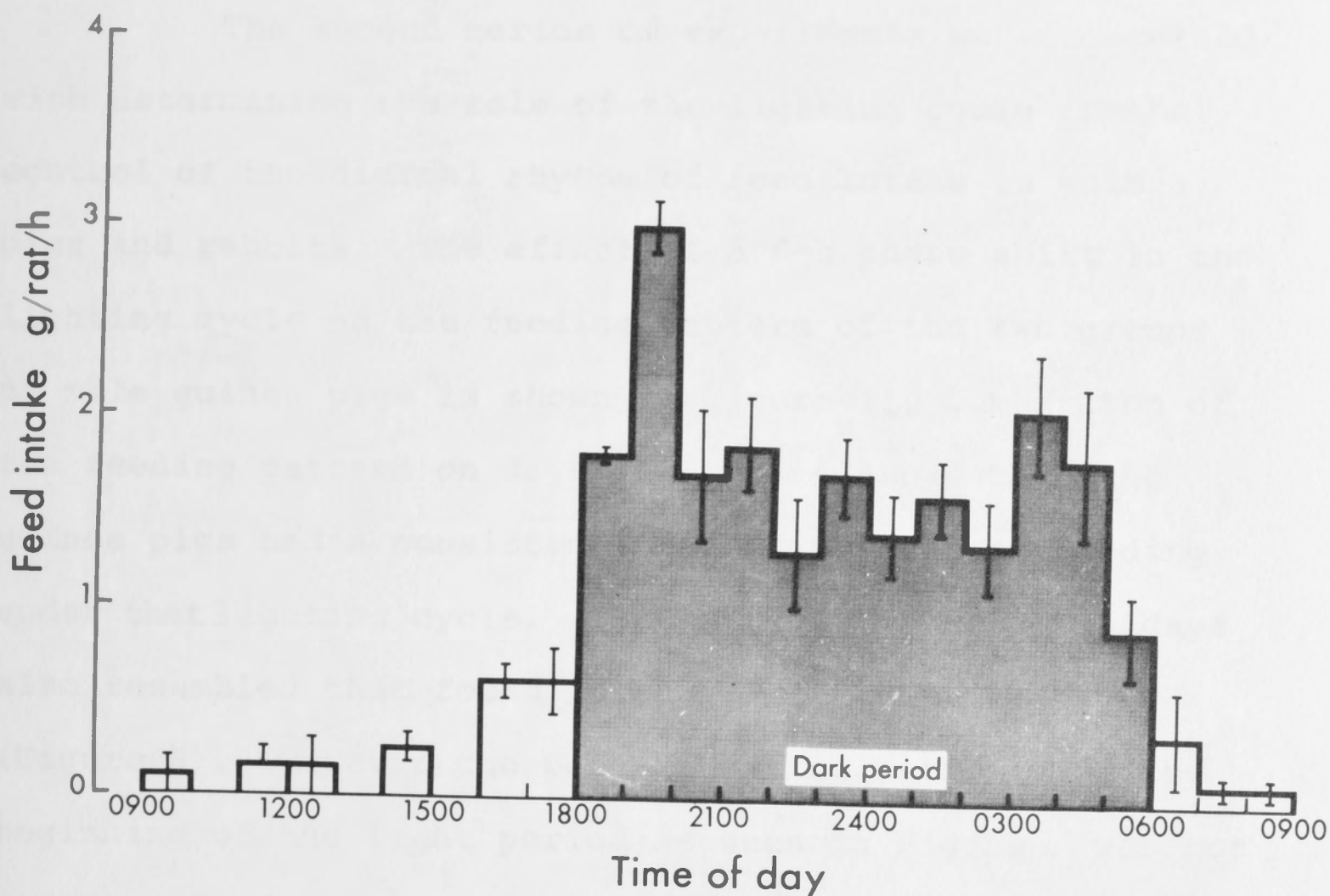


Figure 10. *Diurnal variation in the feed intake of male rats.* The hourly feed intakes of 5 groups of rats, each containing 4 animals, were measured for 24 h. The light period was from 0600 to 1800 h. Values are the mean  $\pm$  S.E.

irregular. This pattern of feeding was similar to that reported by others (Cohn et al., 1970; Edwards et al., 1972; Horton and Sabine, 1973).

The second series of experiments were concerned with determining the role of the lighting cycle in the control of the diurnal rhythm of feed intake in guinea pigs and rabbits. The effect of a 6-h phase shift in the lighting cycle on the feeding pattern of the two groups of male guinea pigs is shown in Figure 11. Comparison of the feeding pattern on days -12 and -4 shows that the guinea pigs had a consistent diurnal pattern of feeding under that lighting cycle. The pattern on these two days also resembled that found in the first experiment (Figure 8). However the peak of eating activity at the beginning of the light period as seen in Figure 11 was not as pronounced as that observed in the first experiment. The feeding pattern 8 and 15 days after the change in the lighting cycle was markedly different from that observed on days -12 and -4. The peak of eating activity observed at the onset of the light period before the 6-h shift in the lighting cycle, was displaced by 6 h to correspond with the change in lighting.

The results obtained with female guinea pigs were similar to those found with males. The feed intake data before and after the phase shift in lighting were analysed by the shifting phase analysis correlation technique described by Horton and West (1976). The values in Table 4 are correlation coefficients calculated by comparing the hourly feed intakes recorded on day -4 with those recorded on days -12, 8 and 15. When the data



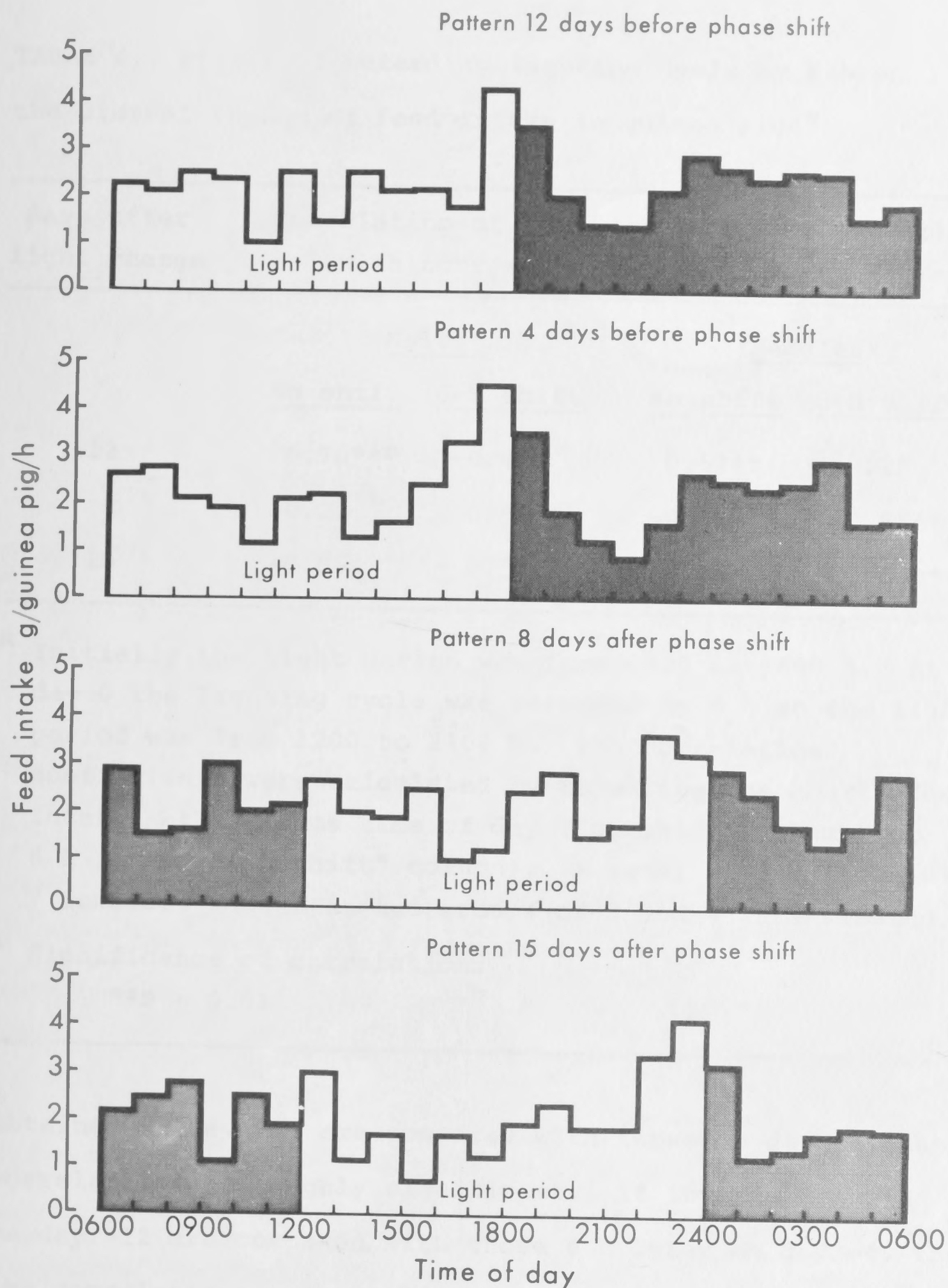


Figure 11. Effect of retarding lighting cycle by 6 h on the diurnal rhythm of feed intake in male guinea pigs. Initially the light period was from 0600 to 1800 h. At day 0 the lighting cycle was retarded by 6 h so that the light period was from 1200 to 2400 h. 12 and 4 days before, and 8 and 15 days after the phase shift the hourly feed intakes of 7 males, housed as groups of 3 and 4, were measured for 24 h.

TABLE 4. Effect of retarding lighting cycle by 6 h on the diurnal rhythm of feed intake in guinea pigs<sup>a</sup>.

Days after light change	Correlation of hourly feeding pattern (r) with corresponding time at day -4			
	<u>Males</u>		<u>Females</u>	
	<u>No shift</u>	<u>6-h shift</u>	<u>No shift</u>	<u>6-h shift</u>
-12	0.76**b	-0.02	0.57**	-0.28
8	-0.21	0.66**	-0.32	0.55**
15	-0.06	0.58**	-0.04	0.56**

<sup>a</sup> Initially the light period was from 0600 to 1800 h. At day 0 the lighting cycle was retarded by 6 h so the light period was from 1200 to 2400 h. The correlation coefficients were calculated by comparing the hourly feed intakes at the same time of day ("no shift" column) or 6 h later ("6-h shift" column). A total of 7 males and 7 females, housed as two groups of 3 and 4 were studied.

<sup>b</sup> Significance of correlation:

\*\*p < 0.01

obtained on day -12 are compared with those on day -4, the correlations are highly significant. If intakes measured on day -12 are compared with those 6 h later on day -4, then the correlations are not significant. Both sexes thus showed a consistent diurnal pattern in feed intake before the change in the lighting cycle was made. There is not a significant correlation between feed consumptions on day -4 and those on days 8 and 15 indicating that the guinea pigs had a different pattern of eating after the 6-h phase shift. However when the feed intakes for day -4 are compared with

those for days 8 and 15, after allowing for the lighting shift of 6 h, significant correlations are obtained for both days. The guinea pigs had thus adjusted their feeding pattern to the new lighting cycle within 8 days of the change.

The effect of the same 6-h phase shift on the diurnal rhythm of feed intake in rabbits is described in Figure 12. The patterns 12 and 4 days before the phase shift are similar to those recorded in the first and second experiments with rabbits (Figure 9). Some shift in the feeding pattern had occurred 8 days after the lighting change and the shift was almost complete on the 15th day. A statistical analysis of the data was carried out in the same way as described for guinea pigs. The results are given in Table 5. When the data obtained on day -12 are compared with those on day -4, the correlation is highly significant. If intakes measured on day -12 are compared with those 6 h later on day -4, then the correlation is not significant. This means that the rabbits showed a consistent diurnal pattern of eating before the change in the lighting cycle was made, confirming the conclusions of the second experiment when the feed consumptions were recorded for 2 consecutive days. The lack of correlation between the feed consumptions on day -4 with those on days 8 and 15 indicates that the rabbits must have had a different pattern of eating after the lighting cycle was changed. When the feed consumptions for day -4 are compared with those for days 8 and 15, after allowing for the lighting shift of 6 h, it is only for day 15 that the correlation is significant. Thus, although there was some change in the



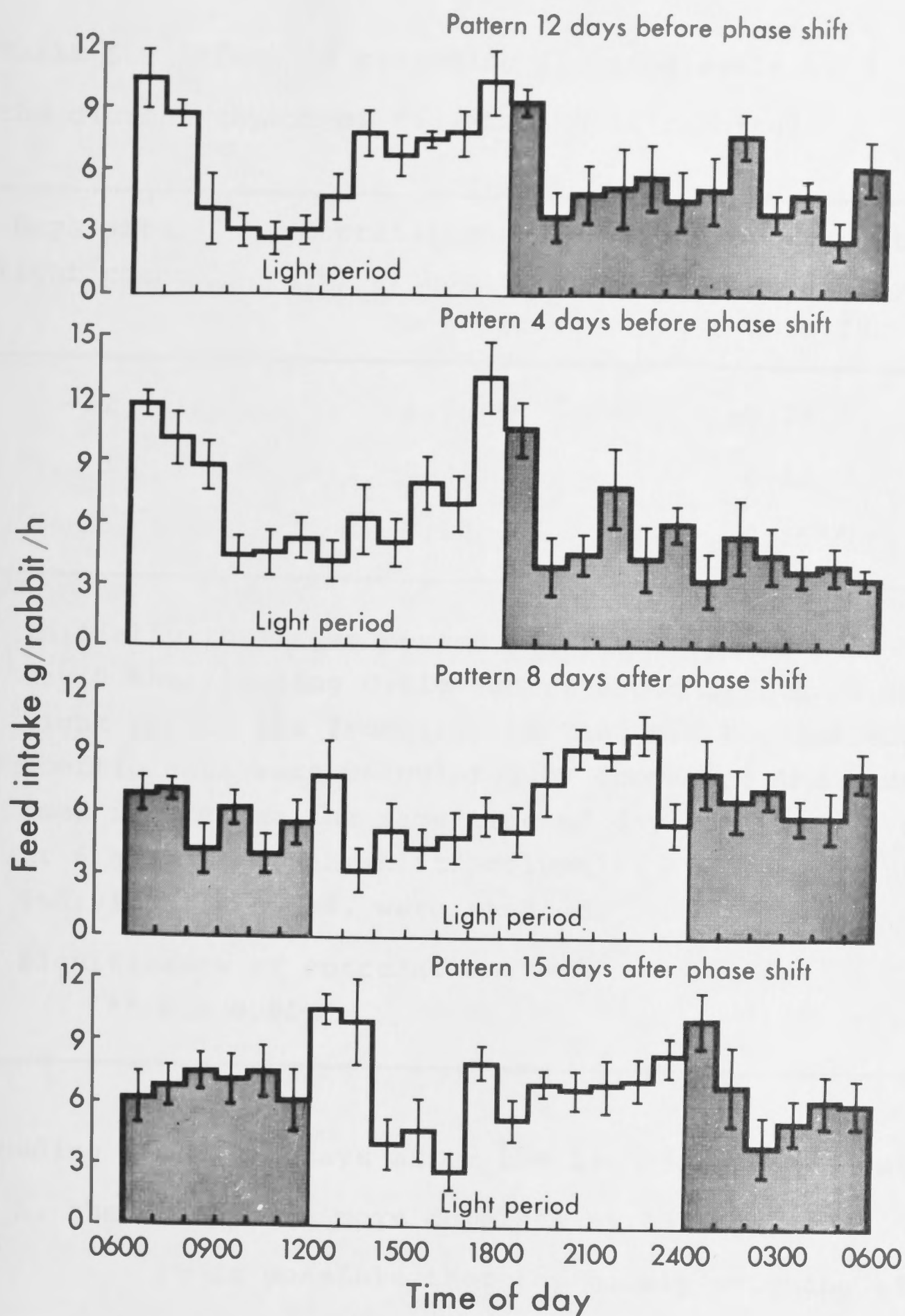


Figure 12. Effect of retarding lighting cycle by 6 h on the diurnal rhythm of feed intake in female rabbits. Initially the light period was from 0600 to 1800 h. At day 0 the lighting cycle was retarded by 6 h so that the light period was from 1200 to 2400 h. 12 and 4 days before, and 8 and 15 days after the phase shift the hourly feed intakes of 9 animals were measured for 24 h. Values are the mean  $\pm$  S.E.

TABLE 5. Effect of retarding lighting cycle by 6 h on the diurnal rhythm of feed intake in rabbits<sup>a</sup>.

Days after light change	Correlation of hourly feeding pattern (r) with corresponding time at day -4	
	No shift	6-h shift
-12	0.76** <sup>b</sup>	-0.29
8	-0.25	0.12
15	-0.10	0.56**

<sup>a</sup> Initially the light period was from 0600 to 1800 h. At day 0 the lighting cycle was retarded by 6 h so that the light period was from 1200 to 2400 h. The correlation coefficients were calculated by comparing the hourly feed intakes at the same time of day ("no shift" column) or 6 h later ("6-h shift" column). A total of 9 females, individually caged, were studied.

<sup>b</sup> Significance of correlation:

\*\*  $P < 0.01$

feeding pattern 8 days after the lighting regime was retarded 6 h, the change was more complete at 15 days.

It is possible that the hourly weighing of feed hoppers may have caused some alteration in feed intake. However, this disturbance must have been of minimal importance because the feeding pattern was independent of the time at which weighing commenced, and similar feed intakes were observed when weighing was carried out at 4- or 6-hourly intervals rather than hourly.

(b) Discussion.

The present studies have confirmed the finding of many other workers that rats maintained under controlled lighting and fed ad libitum consume most of their feed during the dark period. It is valid to directly compare the feeding pattern of the guinea pigs and rabbits with that of the rats because they were maintained under almost identical conditions to the rats and the method of examining the feeding pattern was the same for all three species. Unlike that of the rats, the diurnal rhythm in the feeding pattern of the guinea pigs and rabbits was characterised by several periods of increased eating activity. These occurred at the beginning and end of the light period and in the middle of the dark period. However the rhythm of feed intake in guinea pigs was more pronounced than in the rabbits, particularly with respect to the peak of eating activity in the middle of the dark period. The guinea pigs were also found to adjust their rhythm of feed intake to a phase shift in the lighting cycle more rapidly than rabbits. These differences between the two species may reflect in part behavioural differences associated with maintaining the rabbits in single cages and the guinea pigs in groups of 3-5. Previously it had been assumed that the laboratory rabbit was a continuous (Cizek, 1961; Napier, 1963) or nocturnal (Fox and Laird, 1970) eater. The wild rabbit however has been shown to eat mainly at dawn and dusk (Worden and Leahy, 1962). The results of the present study indicate that there is some similarity in the feeding patterns of the laboratory rabbit and its wild counterpart as periods of increased



eating activity were observed at the time the light period commenced and finished.

The role of the lighting cycle in the control of the feeding pattern of rats has been extensively studied. It has been established that the lighting cycle synchronises but does not generate the diurnal rhythm in feed intake. Thus when the normal light-dark cycle is reversed, complete inversion of the diurnal rhythm of feed intake does not occur until at least 7-9 days later (Zucker, 1971). The maintenance of rats under continuous lighting for about 9 days results in desynchronisation of the rhythm in feed intake (Zucker, 1971; Huber et al., 1973). Such desynchronisation however does not occur in rats maintained under continuous darkness (Huber et al., 1973). Although the guinea pig and rabbit show a markedly different pattern of feeding to the rat, the experiments on the effect of a 6-h phase shift established that the diurnal rhythm of feed intake in both these species is also synchronised by the lighting cycle. Zucker (1971) has concluded that the delay in the adaptation of the rhythm in feed intake to changes in the lighting cycle is indicative of a mechanism which acts to maintain 24-h periodicity despite changes in environmental factors to which the rhythm is synchronised.

In rats the rhythmic changes of cholesterol synthesis in the liver and intestine are associated with parallel changes in the activity of HMG CoA reductase, the rate-limiting enzyme of cholesterol synthesis (Edwards et al., 1972; Shefer et al., 1972b). The role of the lighting cycle and feeding pattern in the control

of the diurnal rhythm in hepatic cholesterol synthesis and HMG CoA reductase activity has been extensively studied in rats. The peak of the rhythms occurs at about the middle of the dark period when rats are fed ad libitum and maintained under 12 h of light and 12 h of dark. However in rats adapted to eating for only a few hours during the light period, the peak of the rhythm occurs several hours later and within the light period (Dugan et al., 1972; Edwards et al., 1972). Thus the cyclic pattern of feed intake and not the lighting cycle per se regulates the timing of the rhythm.

The rhythm persists in rats in which the rate of hepatic cholesterogenesis has been enhanced by cholestyramine feeding (Horton et al., 1970; Shefer et al., 1972b; Weis and Dietschy, 1975) or decreased by cholesterol feeding (Horton et al., 1970; Weis and Dietschy, 1975). The rhythm also persists in fasted rats (Hamprecht et al., 1969; Dugan et al., 1972; Hickman et al., 1972; Weis and Dietschy, 1975) although the rate of cholesterol synthesis is greatly decreased. This demonstrates that the occurrence of the rhythm is independent of feed intake although its magnitude and timing are not.

Recent studies have indicated that a hormonal mechanism, which is influenced by feeding, may regulate the rhythm of hepatic cholesterogenesis. Dugan et al. (1974) have shown that insulin and triiodothyronine together, but not alone, stimulate hepatic HMG CoA reductase activity in rats. The effect of insulin occurs within approximately 2 h whereas that of triiodothyronine

occurs after 2 days. Similar findings have been reported by Huber et al. (1974). However other hormones such as glucagon and hydrocortisone inhibit the stimulatory effect of insulin and triiodothyronine. Dugan et al. (1974) have thus proposed that the diurnal rhythm of HMG CoA reductase in liver is regulated by the relative concentrations of the stimulatory and inhibitory hormones. There is some doubt however concerning the role of adrenalcorticosteroids in the regulation of the rhythm. Recent studies carried out under carefully controlled conditions showed that these hormones do not directly influence hepatic cholesterologenesis in the rat (Nervi and Dietschy, 1974).

Dugan et al. (1974) suggest that the cyclic pattern of feed intake may regulate the timing of the diurnal rhythm in cholesterol synthesis by regulating the relative concentrations of the stimulatory and inhibitory hormones. There is some evidence to support this concept. Thus in rats fed ad libitum there is a diurnal variation in plasma insulin concentration (Kaul and Berdanier, 1972) which is similar to that in HMG CoA reductase activity. Studies with humans have also shown that following ingestion of carbohydrate, insulin secretion increases whereas glucagon secretion decreases (Unger et al., 1963).

It is clear however that further studies are necessary to determine the precise nature of the interaction between cyclic changes in feed intake and the levels of hormones known to influence the rate of cholesterologenesis. The use of the rat for such studies is of limited value because the feeding pattern of this



species is unlike that of other laboratory animals such as the guinea pig and rabbit and also of man. These species show a diurnal rhythm in feeding with multiple periods of increased eating. If feeding directly altered the concentrations of various hormones, then in species such as the guinea pig the levels of hormones such as insulin should rise several times throughout the day. The diurnal rhythm in HMG CoA reductase activity and cholesterol synthesis might therefore be expected to show multiple peaks in species such as the guinea pig. However as shown by the studies described in Part I and Part IV this does not occur. Although guinea pigs showed three periods of increased eating during a 24-h period there was only one peak in hepatic sterologogenesis, which occurred during the dark period. The studies described in Part IV also show that in the guinea pig the peak in the diurnal rhythm of intestinal sterologogenesis occurs about 12 h after that in hepatic sterologogenesis. This finding further indicates that the interaction between the cyclic pattern of feed intake and the diurnal rhythms of sterologogenesis is perhaps more complex than is immediately apparent from studies with rats.

In studies of metabolic regulation and its relevance to the incidence of certain diseases, emphasis has been placed on the frequency of feeding. This applies particularly to the control of plasma cholesterol concentration and the incidence of atherosclerosis (Cohn, 1964; Fábry et al., 1964). In rabbits (Wells et al., 1962) and chickens (Cohn et al., 1961) a change from feeding ad libitum to meal feeding results in a significant

increase in plasma cholesterol concentration. Similarly in humans a reduction in meal frequency leads to enhanced plasma cholesterol concentration (Cohn, 1964; Fábry et al., 1964). Studies with meal-fed rats suggest that the increased plasma cholesterol concentrations may result because of an effect on cholesterol synthesis (Leveille and Hanson, 1965) and not on cholesterol catabolism (Bobek et al., 1972). Clearly, because of the basic difference in the natural feeding pattern of the rat and other species including man, the effect of a change in feeding frequency may affect different aspects of cholesterol metabolism in different species. The present studies suggest that the guinea pig and rabbit would be useful models for further studying this problem.

Ordinary experiments showed that in guinea pig liver slices, the optimum pH for sterologensis was below 7.0 and that much higher concentrations of acetate were needed to achieve maximal rates of sterologensis. Thus further studies were carried out to determine the optimum pH and concentration of acetate for the measurement of sterologensis in the liver, ileum and lung of the guinea pig. The optimum acetate concentration for the measurement of sterol synthesis in rat liver and ileum was similarly determined as was the optimum concentration of mevalonate for the measurement of sterol synthesis from 2-<sup>14</sup>C-mevalonate in guinea pig liver and ileum.

#### (a) Results.

Figure 13 shows the effect of pH of the incubation medium on sterol synthesis in the liver, ileum and lung. Although the liver showed maximum sterol synthesis at pH 6.5, there was little difference in the

PART III. OPTIMAL CONDITIONS FOR THE ASSAY IN VITRO  
OF STEROLOGENESIS IN GUINEA PIG AND RAT TISSUES

As the rate of sterologogenesis in the liver of the guinea pig was found to be very low it was decided to investigate the rates in other tissues. A brief report by Swann and Siperstein (1972) had indicated that the intestine was probably the major site of sterologogenesis in the guinea pig. Although their studies were carried out using tissue slices, details of the assay conditions were not given. The most widely used method for the assay of sterol synthesis in rat liver involves incubation of tissue slices in buffer at pH 7.4 containing 2 mM acetate. However preliminary experiments showed that in guinea pig liver slices, the optimum pH for sterologogenesis was below 7.0 and that much higher concentrations of acetate were needed to achieve maximal rates of sterologogenesis. Thus further studies were carried out to determine the optimum pH and concentration of acetate for the measurement of sterologogenesis in the liver, ileum and lung of the guinea pig. The optimum acetate concentration for the measurement of sterol synthesis in rat liver and ileum was similarly determined as was the optimum concentration of mevalonate for the measurement of sterol synthesis from 2-<sup>14</sup>C-mevalonate in guinea pig liver and ileum.

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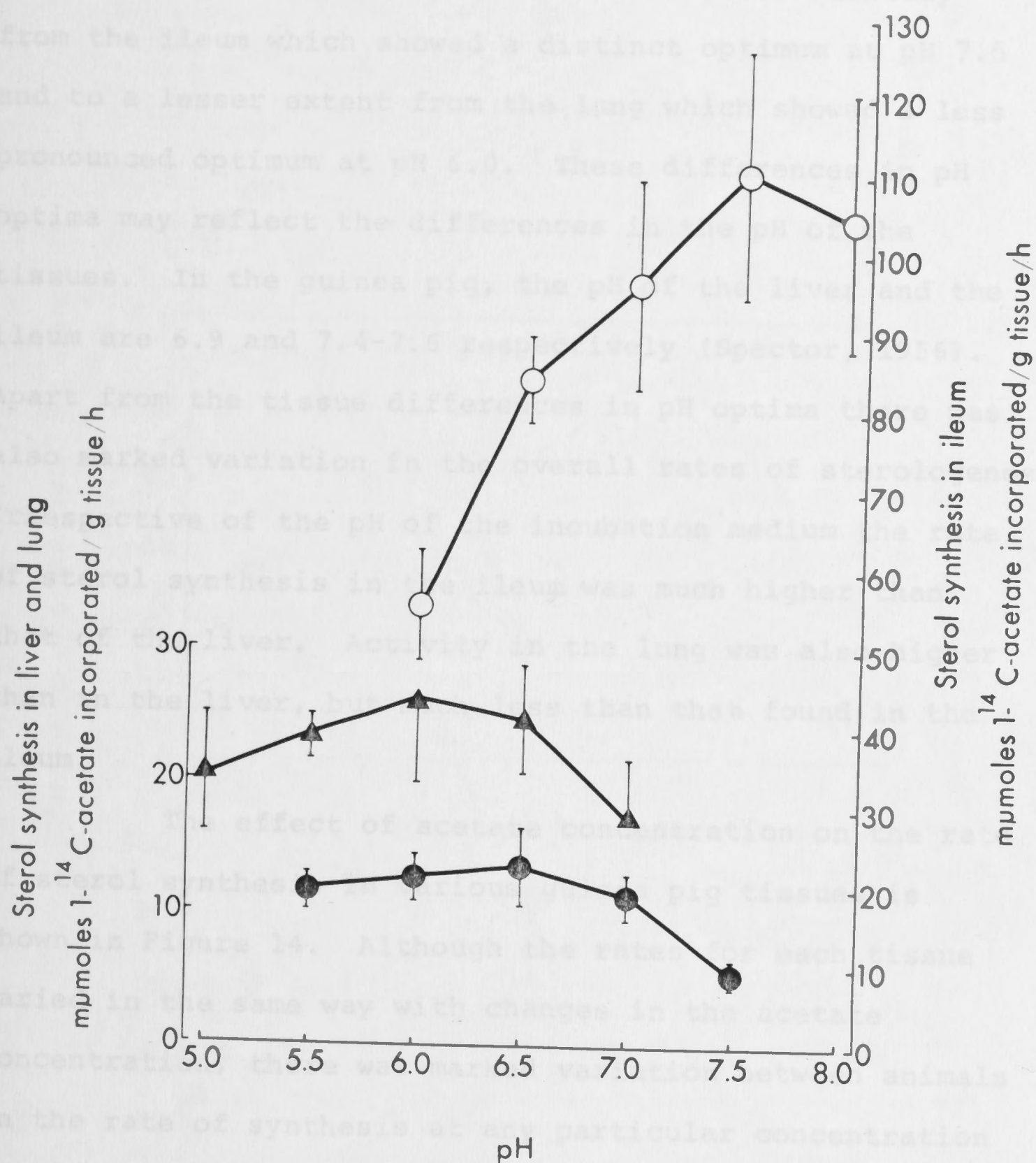


Figure 13. Effect of pH on sterol synthesis in the liver, ileum and lung of male guinea pigs. The incorporation of 1-<sup>14</sup>C-acetate into total digitonin-precipitable sterols by slices of liver (●—●), ileum (○—○) and lung (▲—▲) was measured over the pH range given. Incubations were carried out in triplicate in Krebs-Ringer phosphate buffer. Tissues were obtained from 3 animals killed 3.5–5 h after the commencement of the dark period. Values are the mean ± S.E.

rates between pH 5.5 and 7.0. This differed markedly from the ileum which showed a distinct optimum at pH 7.5 and to a lesser extent from the lung which showed a less pronounced optimum at pH 6.0. These differences in pH optima may reflect the differences in the pH of the tissues. In the guinea pig, the pH of the liver and the ileum are 6.9 and 7.4-7.6 respectively (Spector, 1956). Apart from the tissue differences in pH optima there was also marked variation in the overall rates of sterologogenesis. Irrespective of the pH of the incubation medium the rate of sterol synthesis in the ileum was much higher than that of the liver. Activity in the lung was also higher than in the liver, but much less than that found in the ileum.

The effect of acetate concentration on the rate of sterol synthesis in various guinea pig tissues is shown in Figure 14. Although the rates for each tissue varied in the same way with changes in the acetate concentration, there was marked variation between animals in the rate of synthesis at any particular concentration of acetate. This was particularly so with the liver. The rate of synthesis at each acetate concentration was therefore expressed as a percentage of the activity observed at 20 mM acetate. The mean absolute rates of synthesis (expressed as  $\mu\text{moles/g/h}$ ) for each tissue at 20 mM acetate were: liver ( $65.0 \pm 22.99$ ), ileum ( $102.8 \pm 22.65$ ) and lung ( $22.3 \pm 2.65$ ). As shown in Figure 14A 88% of the maximum sterologenic activity in the liver was obtained at 10 mM acetate. The same result was found for the lung (Figure 14B). In the ileum however (Figure 14C), a

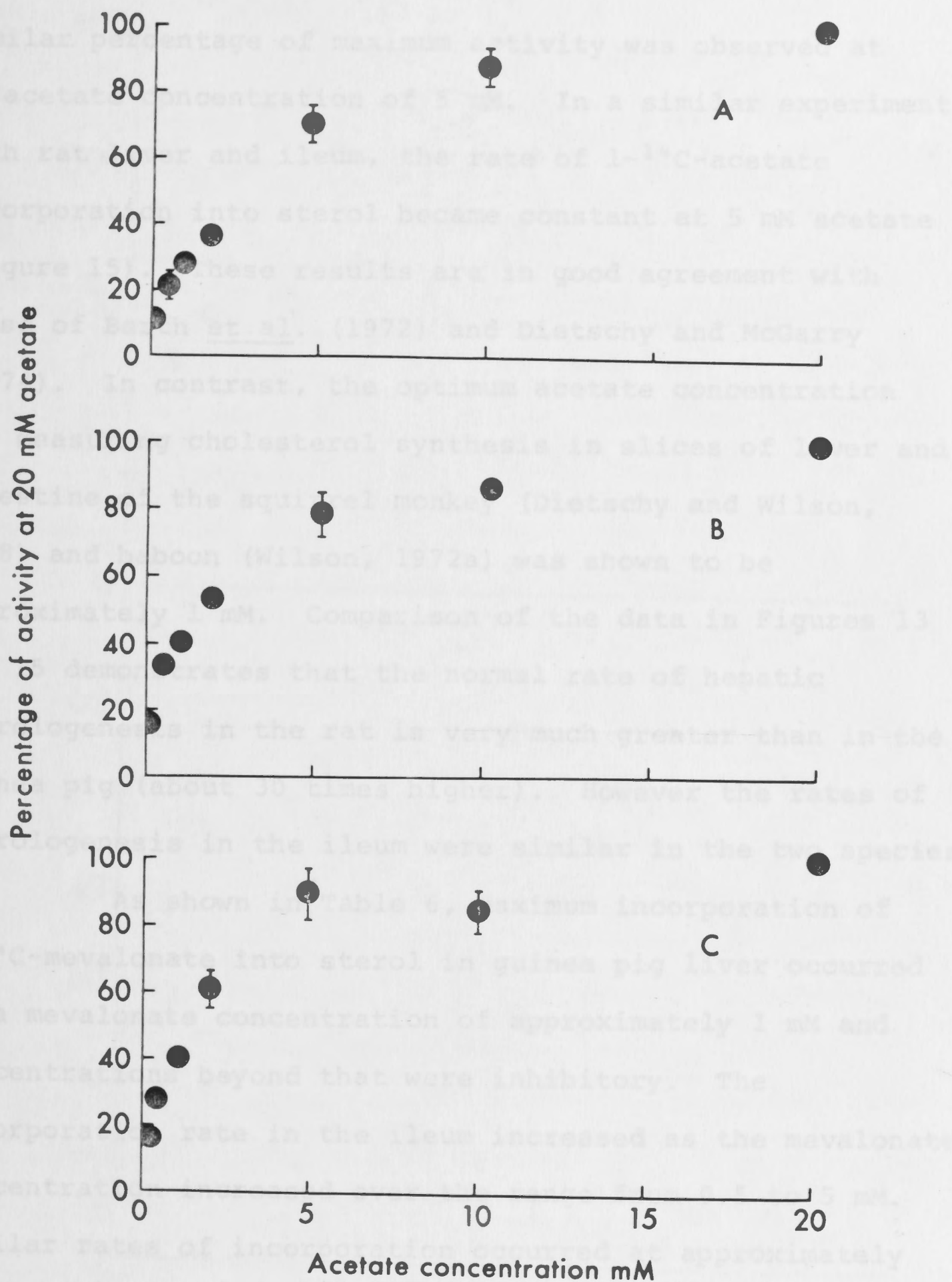


Figure 14. Effect of acetate concentration on sterol synthesis in the liver, ileum and lung of guinea pigs. The incorporation of  $1\text{-}^{14}\text{C}$ -acetate into total digitonin-precipitable sterols by slices of liver (A), lung (B) and ileum (C) was measured at different concentrations of acetate in the incubation medium. Incubations were carried out in triplicate in Krebs-Ringer phosphate buffer. Values are the mean  $\pm$  S.E. of determinations from 4 animals. Separate groups of animals were used for each tissue. Studies were made 3.5-5 h after the commencement of the dark period.



similar percentage of maximum activity was observed at an acetate concentration of 5 mM. In a similar experiment with rat liver and ileum, the rate of 1-<sup>14</sup>C-acetate incorporation into sterol became constant at 5 mM acetate (Figure 15). These results are in good agreement with those of Barth et al. (1972) and Dietschy and McGarry (1974). In contrast, the optimum acetate concentration for measuring cholesterol synthesis in slices of liver and intestine of the squirrel monkey (Dietschy and Wilson, 1968) and baboon (Wilson, 1972a) was shown to be approximately 1 mM. Comparison of the data in Figures 13 and 15 demonstrates that the normal rate of hepatic sterologogenesis in the rat is very much greater than in the guinea pig (about 30 times higher). However the rates of sterologogenesis in the ileum were similar in the two species.

As shown in Table 6, maximum incorporation of 2-<sup>14</sup>C-mevalonate into sterol in guinea pig liver occurred at a mevalonate concentration of approximately 1 mM and concentrations beyond that were inhibitory. The incorporation rate in the ileum increased as the mevalonate concentration increased over the range from 0.5 to 5 mM. Similar rates of incorporation occurred at approximately 5 mM and 9 mM mevalonate.

#### (b) Discussion.

The present study has confirmed the earlier finding of Swann and Siperstein (1972) that in the guinea pig the rate of sterologogenesis in the liver is much less than in the intestine. However it is apparent from a more detailed account of their work (Swann et al., 1975) that the same assay conditions were used for the measurement

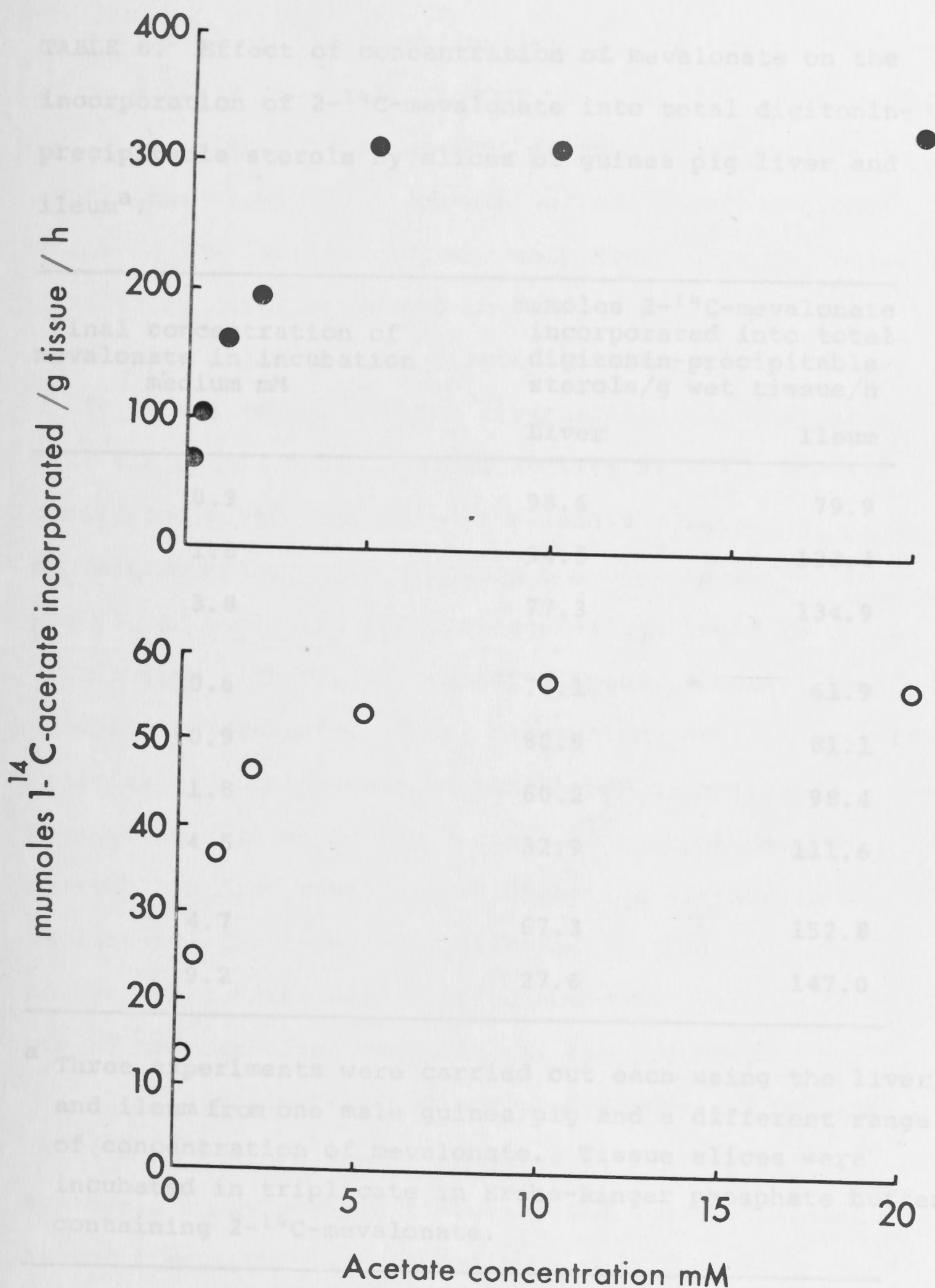


Figure 15. Effect of acetate concentration on sterol synthesis in rat liver and ileum. The incorporation of  $1\text{-}^{14}\text{C}$ -acetate into total digitonin-precipitable sterols by slices of liver (●—●) and ileum (○—○) was measured at different concentrations of acetate in the incubation medium. Incubations were carried out in triplicate in Krebs-Ringer phosphate buffer. 2 male rats were killed at mid-night and their respective tissues pooled.

TABLE 6. Effect of concentration of mevalonate on the incorporation of 2-<sup>14</sup>C-mevalonate into total digitonin-precipitable sterols by slices of guinea pig liver and ileum<sup>a</sup>.

Final concentration of mevalonate in incubation medium mM	μmoles 2- <sup>14</sup> C-mevalonate incorporated into total digitonin-precipitable sterols/g wet tissue/h	
	Liver	Ileum
0.9	98.6	79.9
1.8	94.6	123.4
3.8	77.3	134.9
0.6	76.1	61.9
0.9	80.9	81.1
1.8	60.2	98.4
4.5	32.9	111.6
4.7	67.3	152.8
9.2	27.6	147.0

<sup>a</sup> Three experiments were carried out each using the liver and ileum from one male guinea pig and a different range of concentration of mevalonate. Tissue slices were incubated in triplicate in Krebs-Ringer phosphate buffer containing 2-<sup>14</sup>C-mevalonate.

of sterol synthesis in all the tissues examined. Thus the rates observed by them cannot be directly compared to those found in the present study. This applies particularly to the rates determined in the liver. Swann *et al.* (1975) measured sterol synthesis in all tissues at pH 7.4. In the present work it was found that the



rate of acetate incorporation into sterol by liver slices at pH 7.5 was only half that at pH 6.5.

Although the present study has defined the conditions necessary to obtain maximal incorporation rates of  $^{14}\text{C}$ -labelled acetate into sterol, it is unlikely that these rates represent the absolute rates of sterol synthesis in the various tissues. Dietschy and McGarry (1974), in studies with rat liver slices, demonstrated that the use of radiolabelled acetate as the precursor considerably underestimated the absolute rates of cholesterol synthesis. This occurs because the cytosolic acetyl CoA pool used for cholesterol synthesis is not in equilibrium with the mitochondrial pool of acetyl CoA used for ketogenesis. Thus, alterations of the specific activity of some product of mitochondrial acetyl CoA metabolism such as  $\text{CO}_2$  or ketones, cannot be used to correct the flux rates of radiolabelled acetate into cholesterol for endogenous dilution. It was shown however that these difficulties could be overcome by the use of radiolabelled octanoate as the precursor.

Despite the findings of Dietschy and McGarry (1974), it was decided to continue to measure sterol synthesis in guinea pig tissues using labelled acetate as the precursor. It was believed that the measurement of sterol synthesis from acetate under various experimental manipulations would provide the same information about the mechanisms of control operative in the various tissues as would be gained from the measurement of absolute rates of sterol synthesis under these same conditions. Dietschy and Brown (1974), in

studies with rat liver, have compared the rates of cholesterol synthesis from labelled octanoate and acetate with the HMG CoA reductase activity in the same liver under conditions in which rates of cholesterol synthesis were varied over a 100-fold range. They showed that while there was an excellent correlation between the rate of cholesterol synthesis measured using octanoate as the precursor and the activity of HMG CoA reductase ( $r=0.96$ ), there was also a strong correlation between the incorporation of acetate into cholesterol and HMG CoA reductase activity ( $r=0.79$ ). More recently Swann et al. (1975) have measured sterol synthesis from both acetate and octanoate in several tissues including the liver of both normal and cholesterol-fed guinea pigs. They showed that both the rates of sterol synthesis and the degree of feedback inhibition in the tissues examined were approximately the same with the two substrates.

In the intestine of these species are in marked contrast to those in the human in which the rate in the ileum is about 4 times that in the liver (Dietschy and Gmel, 1971).

In the experiments described in Part III it was found that the guinea pig, like the human, shows much higher rates of sterogenesis in the ileum than in the liver. It was also found, in agreement with Swann and Siperstein (1972) that in the guinea pig the rate of sterol synthesis in the lung exceeded that in the liver. However, sterol synthesis was measured in all tissues at the same time point in the lighting cycle and the rates of sterol synthesis in regions of the intestine other than the ileum were not determined. In the rat (Dietschy

PART IV. RELATIVE RATES OF STEROLOGENESIS IN GUINEA PIG  
AND RAT TISSUES

Considerable information now exists concerning the relative rates of sterologogenesis and more particularly of cholesterologenesis in various tissues of a number of species. In the rat the liver and ileum show the highest rates of cholesterol synthesis - the liver being quantitatively a more important site of synthesis than the intestine (Dietschy and Siperstein, 1967). In monkeys the relative rates of hepatic and intestinal cholesterol synthesis vary markedly with the species although the liver consistently shows a higher rate of cholesterol synthesis than does the intestine (Dietschy and Wilson, 1968; Corey and Hayes, 1974). Baboons also show a higher rate of synthesis in the liver than in the ileum (Wilson, 1972a). The relatively lower rates of cholesterologenesis in the intestine of these species are in marked contrast to those in the human in which the rate in the ileum is about 4 times that in the liver (Dietschy and Gamel, 1971).

In the experiments described in Part III it was found that the guinea pig, like the human, shows much higher rates of sterologogenesis in the ileum than in the liver. It was also found, in agreement with Swann and Siperstein (1972) that in the guinea pig the rate of sterol synthesis in the lung exceeded that in the liver. However, sterol synthesis was measured in all tissues at the same time point in the lighting cycle and the rates of sterol synthesis in regions of the intestine other than the ileum were not determined. In the rat (Dietschy



and Siperstein, 1967), squirrel monkey (Dietschy and Wilson, 1968) and human (Dietschy and Gamel, 1971) the rate of sterologogenesis varies markedly in different regions of the gastrointestinal tract. In rats both hepatic and intestinal sterologogenesis show a marked diurnal variation (Edwards et al., 1972). Thus the rates observed in the experiments described in Part III cannot be used to assess the quantitative significance of sterol synthesis by whole organs, particularly the liver and intestine.

Studies have therefore been carried out to examine the rates of sterol synthesis in the liver, ileum and lung of the guinea pig at regular intervals throughout the day. The rates of sterologogenesis in various regions of the gastrointestinal tract and in several other tissues were also determined as was the effect of age on the relative rates of hepatic and intestinal sterologogenesis. A comparative study on the diurnal variation in sterol synthesis in rat liver and ileum was also carried out.

(a) Results.

The results of the experiment in which sterol synthesis was measured in the liver, ileum and lung of male guinea pigs at 6-hourly intervals over an 18-h period are shown in Figure 16. At all four time points the rate of sterol synthesis in the ileum was much higher than that in the liver and lung. The lung in turn consistently showed a higher rate of sterologogenesis than the liver. However the extent of difference between the three tissues in their rates of sterol synthesis varied markedly with the time of day. This was particularly so with the ileum and liver. At 2100 h, the rate of synthesis in the ileum

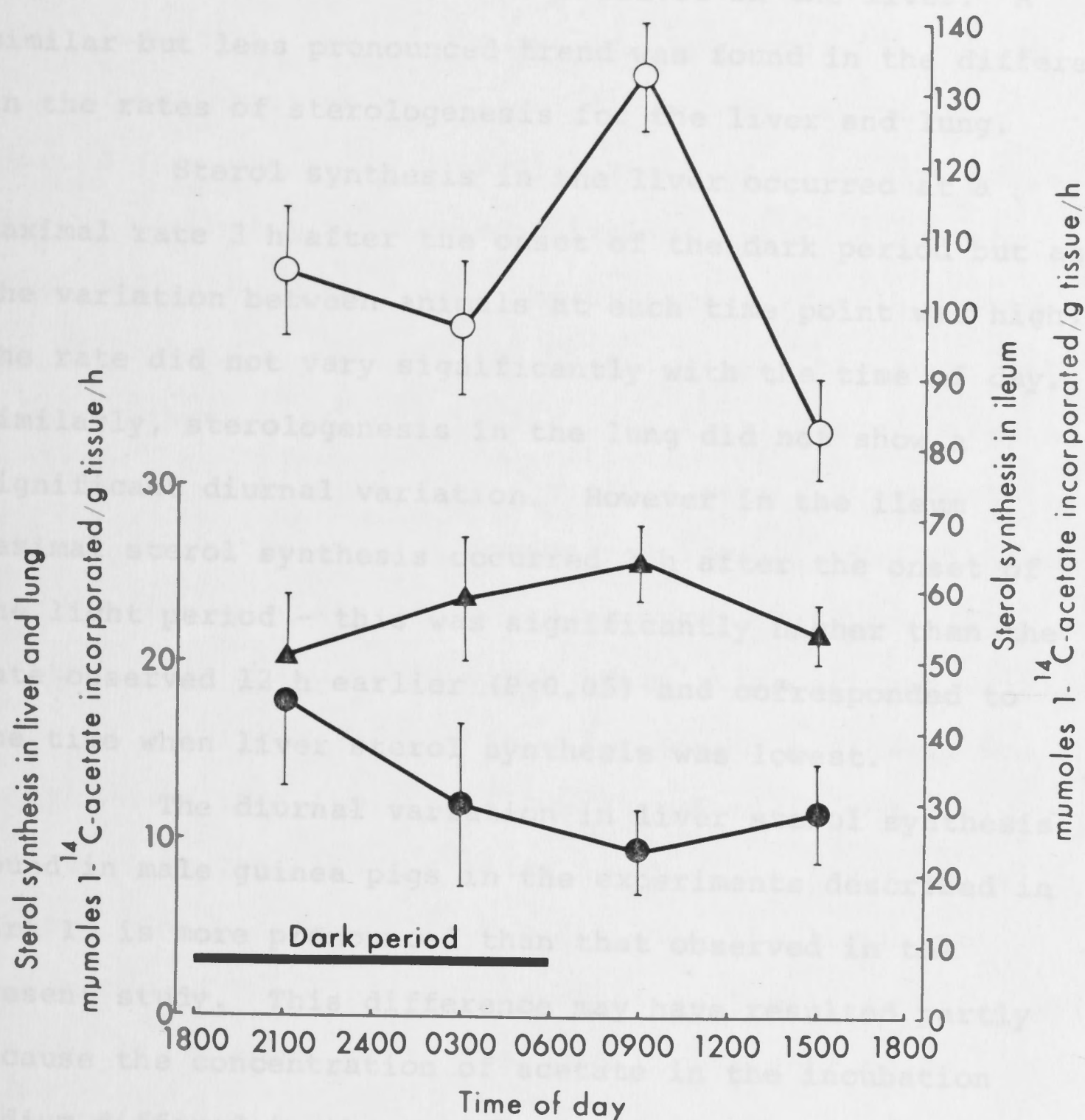


Figure 16. Diurnal variation in sterol synthesis in the liver, ileum and lung of male guinea pigs. Groups of animals were killed at 6-hourly intervals and the incorporation of 1-<sup>14</sup>C-acetate into total digitonin-precipitable sterols by slices of liver (●—●), ileum (○—○) and lung (▲—▲) measured. Incubations were carried out in triplicate in Krebs-Ringer phosphate buffer. At both 2100 and 0900 h 10 animals were used while at 0300 and 1500 h, there were 7 animals. Values are the mean±S.E.

was 6 times that in the liver while 12 h later it was 14 times higher than the rate observed in the liver. A similar but less pronounced trend was found in the differences in the rates of sterologogenesis for the liver and lung.

Sterol synthesis in the liver occurred at a maximal rate 3 h after the onset of the dark period but as the variation between animals at each time point was high, the rate did not vary significantly with the time of day. Similarly, sterologogenesis in the lung did not show a significant diurnal variation. However in the ileum maximal sterol synthesis occurred 3 h after the onset of the light period - this was significantly higher than the rate observed 12 h earlier ( $P < 0.05$ ) and corresponded to the time when liver sterol synthesis was lowest.

The diurnal variation in liver sterol synthesis found in male guinea pigs in the experiments described in Part I, is more pronounced than that observed in the present study. This difference may have resulted partly because the concentration of acetate in the incubation medium differed in the two studies. In the experiments in Part I the incubation medium contained 2 mM acetate while in all subsequent studies 10 mM acetate was used, this being the concentration at which the incorporation of  $1\text{-}^{14}\text{C}$ -acetate into sterol occurred at near maximal rates. Thus any change in the size of the endogenous acetyl CoA pool throughout the day would have had a more pronounced influence on the apparent rate of sterol synthesis when the concentration of exogenous acetate was 2 mM and not 10 mM.



In rats fed ad libitum and maintained under controlled lighting with equal periods of light and dark, the diurnal rhythms of cholesterol synthesis in both the liver and intestine peak about the middle of the dark period (Edwards et al., 1972). These studies were carried out by measuring the incorporation of labelled acetate into cholesterol in vivo. The present studies, carried out in vitro have shown that in the guinea pig the respective diurnal rhythms of hepatic and intestinal sterologogenesis peak about 12 h apart. It therefore seemed important to determine whether the different results obtained with the two species were related to the measurement of sterol synthesis in vitro or in vivo. Thus an experiment with male rats was carried out in which sterol synthesis was measured in the liver and ileum at mid-night and mid-day using assay conditions similar to those in the study with guinea pigs. As shown in Table 7 both the liver and ileum showed their highest rates of synthesis at mid-night. In the liver there was a 3.8-fold increase in sterol synthesis at mid-night while in the ileum a 1.7-fold increase was observed. However only for the liver was the difference between the mid-night and mid-day values significant ( $P < 0.01$ ). These results confirm those obtained by Edwards et al. (1972) using the in vivo technique. The guinea pig and rat therefore show a genuine species difference in their diurnal rhythms of hepatic and intestinal sterologogenesis.

To quantitate sterol synthesis in the liver, ileum and lung differences in organ weights must be taken into account. The ileum, which was taken to be the third

TABLE 7. Rates of sterol synthesis in the liver and ileum of male rats at mid-night and mid-day.

Time of day	Sterol synthesis <sup>a</sup>	
	Liver	Ileum
Mid-night (5) <sup>b</sup>	589.8±78.93 <sup>c</sup>	143.1±36.64**
Mid-day (5)	156.7±33.20**	84.5±17.21

<sup>a</sup> Tissue slices were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-<sup>14</sup>C-acetate. Sterol synthesis is expressed as  $\mu$ moles 1-<sup>14</sup>C-acetate incorporated into total digitonin-precipitable sterols/g wet tissue/h.

<sup>b</sup> Number of animals studied.

<sup>c</sup> Values are the mean±S.E.

Comparisons by Student's t-test with the mid-night value for liver:

\*\* P<0.01

of the small intestine proximal to the ileocaecal junction, weighed approximately one sixth that of the liver. From the data at the various time points in Figure 16 the total sterol synthesis in each organ, expressed as  $\mu$ moles/organ/24 h was calculated to be: liver, 9.1; lung, 2.1 and ileum, 12.8. The ileum alone therefore produces more sterol than does the whole liver. This contrasts greatly to the rat and squirrel monkey in which the liver is quantitatively a much more important site of sterologogenesis than the ileum (Dietschy and Siperstein, 1967; Dietschy and Wilson, 1968). Although total sterol synthesis in the lung was much less than that in the liver and ileum, the lung of the guinea pig is a much more active region

TABLE 8. Rates of sterol synthesis in liver and gastrointestinal tract of guinea pigs<sup>a</sup>.

	Sterol synthesis <sup>b</sup>	
	Male	Female
Number of animals	4	6
Liver	31.0±12.62 <sup>c</sup>	21.3±13.85
Stomach	29.3±3.28	25.3±2.83
Duodenum	55.7±6.02	52.0±8.87
Jejunum	52.3±5.80	41.0±7.37
Ileum	114.0±13.88**	65.7±8.03*
Transverse colon	15.3±1.78	6.0±0.92

<sup>a</sup> Determined in the same animals, 3.5-5 h after the commencement of the dark period.

<sup>b</sup> Tissue slices were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-<sup>14</sup>C-acetate. Sterol synthesis is expressed as μmoles of 1-<sup>14</sup>C-acetate incorporated into total digitonin-precipitable sterols/g wet tissue/h.

<sup>c</sup> Values are the mean±S.E.  
Comparison by Student's t-test with corresponding value for liver:

\*P<0.05      \*\*P<0.01

of sterologogenesis than it is in either the rat or squirrel monkey (Dietschy and Siperstein, 1967; Dietschy and Wilson, 1968).

The relative rates of sterol synthesis in the liver and various regions of the gastrointestinal tract of male and female guinea pigs are described in Table 8. In both sexes the rates in the liver showed high variation



between animals. For example of the 6 females studied, 5 showed a rate of hepatic sterol synthesis which was less than 15  $\mu\text{moles/g/h}$  while in one of the females the rate in the liver was 90  $\mu\text{moles/g/h}$ . An analysis of variance confirmed that the variation between animals was greater than that between the replicates for each animal (females,  $P < 0.005$ ; males,  $P < 0.025$ ). As the guinea pigs were killed at the time in the lighting cycle when the rate of sterol synthesis in the liver was maximal and that in the ileum near minimal, the rates for the various regions of gastrointestinal tract probably represent an underestimation of their average daily rate of sterol synthesis relative to that in the liver. Despite this, all regions of the small intestine, particularly the ileum, exhibited higher rates of sterol synthesis than the liver. The stomach was found to synthesise sterol at rates comparable to those of the liver. However the weight of the liver was on average 8 times that of the stomach making the latter quantitatively less important. In both males and females the rate of sterol synthesis in the transverse colon was much less than that in other regions of the gastrointestinal tract examined and also less than that in the liver. It is clear, however, from the data given in Figure 16 and in Table 8 that in the guinea pig the gastrointestinal tract makes a greater contribution to total body sterol synthesis than does the liver.

The effect of age on the relative rates of hepatic and intestinal sterologogenesis in male guinea pigs is shown in Table 9. The incorporation of 1- $^{14}\text{C}$ -acetate into sterol by the liver did not differ significantly

TABLE 9. Rates of sterol synthesis in the liver and ileum of male guinea pigs of different ages.

	Age (weeks)	
	7	29
Number of animals	4	3
Body weight g	519±23.1 <sup>b</sup>	1016±80.2
Sterol synthesis <sup>a</sup> :		
From 1- <sup>14</sup> C-acetate		
Liver	28.8±3.10	24.3±4.22
Ileum	179.0±5.81	123.8±6.86**
From 2- <sup>14</sup> C-mevalonate		
Liver	70.8±3.49	53.6±6.45
Ileum	194.4±14.14	153.8±3.82

<sup>a</sup> Slices of liver and ileum were incubated in triplicate in Krebs-Ringer phosphate buffer containing either 1-<sup>14</sup>C-acetate or 2-<sup>14</sup>C-mevalonate. Sterol synthesis is expressed as  $\mu$ moles of 1-<sup>14</sup>C-acetate or 2-<sup>14</sup>C-mevalonate incorporated into total digitonin-precipitable sterols/g wet tissue/h.

<sup>b</sup> Values are the mean±S.E.

Comparison by Student's t-test with corresponding value for animals aged 7 weeks:

\*\*P<0.01

between the animals aged 7 weeks and those aged 29 weeks. However the incorporation of acetate into sterol by the ileum was significantly less in the older animals (P<0.01). The incorporation of mevalonate into sterol by both the liver and ileum was lower in the older animals. This difference almost attained statistical significance in both tissues (liver, P=0.051; ileum, P=0.062).

TABLE 10. Rates of sterol synthesis in various tissues of guinea pigs<sup>a</sup>.

	Sterol synthesis <sup>b</sup>
Skin (2) <sup>c</sup>	2.1 <sup>d</sup>
Thigh muscle (2)	<0.1
Epididymal adipose tissue (2)	0.1
Adrenal (3)	27.9
Testis (2)	9.3

<sup>a</sup> Determined 3.5-5 h after the commencement of the dark period.

<sup>b</sup> Tissue slices were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-<sup>14</sup>C-acetate. Sterol synthesis is expressed as  $\mu$ moles of 1-<sup>14</sup>C-acetate incorporated into total digitonin-precipitable sterols/g wet tissue/h.

<sup>c</sup> Number of animals studied. The tissues were not all derived from the same animals.

<sup>d</sup> Mean values.

Preliminary measurements of sterol synthesis in several other tissues of the guinea pig were also made. As shown in Table 10, the rate of sterologogenesis in the skin, thigh muscle and epididymal adipose tissue was extremely low. Thus although these tissues constitute a substantial portion of the total body mass, their contribution to total body sterol synthesis would be small compared to that of the liver and intestine. The testis and adrenal showed similar rates of sterol synthesis to the liver. However total sterol synthesis in these



tissues would be relatively small. As sterol synthesis in all tissues was measured in only 2 or 3 animals, further studies would be needed to substantiate these results.

(b) Discussion.

In these studies the rate of incorporation of labelled acetate into total digitonin-precipitable sterols by each tissue has been taken as a measure of the inherent rate of sterol synthesis of that tissue. No attempt was made to determine the possible differences between tissues in either the rate of entry of acetate into cells or the rate of acetyl CoA formation. However it is unlikely that such effects could account for the marked differences in the rates of hepatic and intestinal sterologogenesis in guinea pigs because when similar conditions of assay were used in rats, the rate of sterol synthesis in the liver was higher than in the intestine, this being the reverse of differences found in guinea pigs. Furthermore if the entry of acetate into cells or the rate of acetyl CoA formation had differed between tissues then those tissues which showed similar rates of sterol synthesis should also have showed similar rates of fatty acid synthesis. As shown by studies described in Part V this did not occur. Although the liver and lung showed similar rates of incorporation of labelled acetate into sterols, the rate of acetate incorporation into fatty acids by the lung was about 10 times that by the liver.

The wide difference observed in vitro between the rates of hepatic and intestinal sterologogenesis in the guinea pig has also been shown in vivo. Schwenk et al. (1955) reported that in guinea pigs the incorporation

rate of labelled acetate into sterols in vivo by the gastrointestinal tract was about 10 times higher than that shown by the liver. Dietschy and Siperstein (1967) showed that in rats the rate of sterologogenesis in the liver exceeded that in the ileum in vitro. This finding was confirmed in the present studies. In contrast, most (Edwards et al., 1972; Chevallier and Magot, 1975) but not all (Sanghvi et al., 1974) studies carried out in vivo have shown that in rats the rate of sterologogenesis in the intestine exceeds that in the liver. The higher intestinal rates observed in vivo, however, may result because labelled sterol, synthesised in the liver, enters the bile and subsequently the intestine where it is reabsorbed. Thus it is not possible to distinguish between sterol synthesised by the intestine and that derived originally from the liver. This limitation of the in vivo technique would apply much more to studies with rats than with guinea pigs because under in vitro conditions the rate of sterologogenesis in rat liver is about 30 times that in guinea pig liver. In rats, depending on the time of day and the age of the animal, the specific activity of HMG CoA reductase in the liver is usually higher than in the intestine (Shefer et al., 1972a,b). HMG CoA reductase has been shown to be the rate-limiting enzyme of liver sterol synthesis in rats (Siperstein and Fagan, 1966; Slakey et al., 1972) and is also considered to be the rate-limiting enzyme in intestinal sterologogenesis (Dietschy, 1968). Thus the relative rates of sterologogenesis observed in vitro in the various tissues probably closely reflect the differences which occur in the whole animal.

The high variation in the rates of sterologogenesis in guinea pig tissues, particularly in the liver, genuinely reflected marked individual variation between animals as there was good agreement between replicates from the same animal. Swann et al. (1975) have also found marked individual variation in the rate of hepatic sterologogenesis in guinea pigs. High individual variation in hepatic cholesterol synthesis in the cebus monkey (MacNintch et al., 1967) and baboon (Wilson, 1972a) has also been reported. Such variation is not found in the laboratory rat possibly because of its prolonged selective breeding.

In rats, cholesterol synthesis in the liver decreases markedly with age (Yamamoto and Yamamura, 1971). Studies by Hamprecht et al. (1971a) and Shefer et al. (1972a) have further shown that in rats there is a decrease with age in the specific activity of HMG CoA reductase in the liver. However the activity of intestinal HMG CoA reductase in rats does not decrease with age (Shefer et al., 1972a). The present studies have shown that in guinea pigs there is a greater effect of age on intestinal than on hepatic sterologogenesis. The lower incorporation of mevalonate into sterol by the liver and ileum in the older guinea pigs suggests that the activity of an enzyme subsequent to HMG CoA reductase may also decrease with age.

The difference in the diurnal rhythms of hepatic and intestinal sterologogenesis shown by the guinea pig and rat may be explained by the marked difference in the feeding pattern of the two species. As shown in Part II there were three periods of increased eating in the diurnal rhythm of feed intake in guinea pigs. These



occurred at the beginning and end of the light period and in the middle of the dark period. In contrast there was only one major period of eating in the diurnal rhythm of feed intake of rats. This occurred during the dark period. In rats fed ad libitum the diurnal rhythms of hepatic and intestinal sterologogenesis both peak about the middle of the dark period (Edwards et al., 1972). However in rats adapted to a restricted feeding schedule the timing of the rhythms in the liver and intestine is different. Thus Edwards et al. (1972) showed that in rats maintained under 12 h of light and 12 h of dark and fed for only 4 h during the light period, maximum sterol synthesis in the liver occurred 9 h after the presentation of feed. However in the ileum the maximum occurred after only 3 h while in the jejunum the maximum was essentially constant from 3 to 12 h after the presentation of feed. While there is no clear explanation for the different effects in the liver and intestine, the results of this study imply that in those species with a feeding pattern different to that shown by rats fed ad libitum, the timing of the diurnal rhythm of sterologogenesis should be expected to differ in the various tissues.

In the rat (Dietschy and Siperstein, 1967), squirrel monkey (Dietschy and Wilson, 1968) and human (Dietschy and Gamel, 1971) sterologogenesis in the liver, intestine and various other tissues is considered a direct measure of cholesterologenesis because the principal sterol produced is cholesterol. This also applies to sterol synthesis in the liver of the guinea pig because at least 70% of the sterol synthesised is cholesterol. However it

cannot be applied to intestinal sterologogenesis in this species because less than 5% of the sterol synthesised in the small intestine is cholesterol. The principal sterols produced are lathosterol and 7-dehydrocholesterol (Ockner and Laster, 1966). The results of these studies, which were carried out by examining the incorporation of labelled acetate into various sterols by mucosal scrapings from the small intestine, confirmed the findings of Schwenk et al. (1955) which showed that in vivo, guinea pig intestine incorporated labelled acetate primarily into sterols other than cholesterol. Thus although the present studies have established that in guinea pigs the gastrointestinal tract makes a much greater contribution to total body sterol synthesis than the liver, the quantity of cholesterol produced by the liver probably greatly exceeds that of the gastrointestinal tract.

Despite the difference in the type of sterol synthesised by guinea pig liver and intestine, the principal sterol contained in both tissues is cholesterol (Ockner and Laster, 1966). Much of the intestinal cholesterol is probably derived from the diet and bile. Only a small proportion of the sterol in the intestinal mucosa is lathosterol and 7-dehydrocholesterol. Wells et al. (1955) have shown that the faeces of guinea pigs contain large quantities of lathosterol and 7-dehydrocholesterol and that these originated mostly in the intestinal mucosa.

The study of the relative rates of cholesterologenesis in various tissues is of significance in determining the likely endogenous sources of plasma cholesterol. In the rat (Dietschy and Siperstein, 1967) and squirrel monkey

(Dietschy and Wilson, 1968) the liver and small intestine synthesise about 90% of the total cholesterol produced by the body. Although the studies of Morris et al. (1957) suggest that in rats the liver is the major endogenous source of plasma cholesterol, Lindsey and Wilson (1965) have shown that in this species cholesterol synthesised in the intestine also reaches the circulation. In the squirrel monkey the intestine has been shown to contribute significantly to the plasma cholesterol pool (Wilson, 1968). It is believed that in the human the extrahepatic tissues are the major endogenous source of plasma cholesterol (Cox et al., 1963). In the guinea pig however, the liver is probably the major endogenous source of plasma cholesterol.

Although the principal sterols synthesised in the small intestine of the human and guinea pig differ, the rate of sterologogenesis in the ileum greatly exceeds that in the liver in both species. This differs from the relative rates of hepatic and intestinal sterologogenesis shown by the rat, squirrel monkey and baboon. It therefore seemed important to determine whether the mechanisms of control of sterologogenesis which have been elucidated in the liver and intestine of the human were operative in the guinea pig. The results of these studies are described in Part V.

negative feedback system similar to that in the rat and squirrel monkey.

The control of sterologogenesis in tissues of the guinea pig, particularly the extrahepatic tissues, has not been the subject of detailed study. However it is known



PART V. EFFECTS OF CHOLESTEROL AND CHOLESTYRAMINE FEEDING  
AND OF FASTING ON STEROLOGENESIS IN GUINEA PIG TISSUES.

There is now substantial evidence from studies with several mammalian species including man which suggests that hepatic and intestinal sterologogenesis and more particularly cholesterologenesis are subject to different mechanisms of control (Dietschy and Wilson, 1970a). In the rat (Dietschy and Siperstein, 1965, 1967; Cayen, 1969), squirrel monkey (Dietschy and Wilson, 1968) and man (Bhattathiry and Siperstein, 1963; Dietschy and Gamel, 1971), cholesterol feeding markedly inhibits hepatic cholesterologenesis while having relatively little effect on cholesterol synthesis in the intestine. Similarly fasting has a much greater inhibitory action on hepatic than on intestinal cholesterologenesis in the rat (Dietschy and Siperstein, 1967) and squirrel monkey (Dietschy and Wilson, 1968). In contrast biliary diversion in these species has a much greater stimulatory effect on intestinal cholesterol synthesis than it has on cholesterol synthesis in the liver (Dietschy, 1968; Dietschy and Wilson, 1968; Weis and Dietschy, 1969). In patients with biliary obstruction, a marked increase in cholesterol synthesis in the intestine has been reported (Dietschy and Gamel, 1971) indicating the presence in man of a bile acid negative feedback system similar to that in the rat and squirrel monkey.

The control of sterologogenesis in tissues of the guinea pig, particularly the extrahepatic tissues, has not been the subject of detailed study. However it is known

that hepatic sterol synthesis in the guinea pig is markedly depressed by fasting (Sauer, 1960) and by feeding either cholesterol (Beher et al., 1963; Swann and Siperstein, 1972) or various bile acids (Beher et al., 1963). There is also evidence that the cholesterol negative feedback system operates in a wide range of guinea pig tissues (Swann and Siperstein, 1972). The effect of the interruption of the enterohepatic circulation of bile acids on sterol synthesis in tissues of the guinea pig apparently has not been previously determined. However a brief report by Laster et al. (1966) indicated that intestinal sterologogenesis in the guinea pig could be markedly inhibited by bile salts in vitro.

In the studies described in Part IV, it was shown that in guinea pigs the rate of sterologogenesis in the liver was very low compared to that in the small intestine. The present studies were therefore carried out to examine the factors which control sterol synthesis in guinea pig tissues. This involved the measurement of the rate of sterol synthesis in vitro in the liver, ileum and lung of guinea pigs fed diets containing either cholesterol or cholestyramine or which had been fasted for 24 h.

(a) Results.

In the first experiment the effect of cholesterol and cholestyramine feeding on sterol synthesis in the liver, ileum and lung of female guinea pigs was examined. As shown in Table 11 the body weights of the guinea pigs fed the cholesterol and cholestyramine diets did not differ significantly from those of guinea pigs fed the plain diet.

TABLE 11. Effect of cholesterol and cholestyramine feeding on body weights of female and male guinea pigs<sup>a</sup>.

Sex	Diet	Number of animals	Days on diet	Body weight g	
				Initial	Final
Female	Plain	6	15	770±20.7 <sup>b</sup>	783±22.8
	Cholesterol	6	16	756±38.4	712±52.9
	Cholestyramine	6	14	770±29.5	763±28.9
Male	Plain	4	14	709±18.3	762±28.4
	Cholesterol	4	15	736±31.3	755±37.3
	Cholestyramine	4	12	736±21.6	730±34.2

<sup>a</sup> Female and male guinea pigs were fed the plain diet or a diet containing either 0.24% w/w cholesterol or 2.5% w/w cholestyramine. Individual body weights were recorded at the beginning and end of the experiments.

<sup>b</sup> Values are the mean±S.E.

Differences between either the initial and final value for each group or between groups at both the initial and final stages are not significant.



The feed consumption of the guinea pigs, which did not differ between the animals on the different diets, was approximately 60 g/kg bw/day. All animals were killed approximately 4 h after the commencement of the dark period.

The effects of feeding the diets containing added cholesterol and cholestyramine on sterol synthesis in the liver, ileum and lung are shown in Table 12. Cholesterol feeding significantly decreased the rate of sterologogenesis in the ileum ( $P < 0.05$ ) but not in the liver and lung. The most pronounced effect of cholestyramine feeding was on the rate of hepatic sterologogenesis which was 13 times higher in the cholestyramine-fed animals than in those fed the plain diet ( $P < 0.001$ ). In contrast the rate of sterol synthesis in the ileum was not significantly altered while in the lung it was increased by 81% ( $P < 0.05$ ).

In the experiment with females no attempt was made to determine to what extent the changes which occurred in sterol synthesis in the animals fed cholesterol and cholestyramine may have been due to a direct effect of these treatments on the size of the acetyl CoA pool. Therefore a similar experiment was carried out in which both sterol and fatty acid synthesis were measured in the same incubations. In this experiment male guinea pigs were used. As in the experiment with females, the body weights of the animals fed the cholesterol and cholestyramine diets did not differ significantly from those of guinea pigs fed the plain diet (Table 11). The feed intake of the males receiving the three respective diets was also approximately 60 g/kg bw/day. As in the first experiment all animals were killed approximately

TABLE 12. Effect of cholesterol and cholestyramine feeding on sterol synthesis in the liver, ileum and lung of female guinea pigs<sup>a</sup>.

Diet	Number of animals	Sterol synthesis		
		Liver	Ileum	Lung
Plain	6	13.1±4.40 <sup>b</sup>	86.4±9.79	12.8±1.47
Cholesterol	6	3.6±2.94	54.9±9.83*	11.5±2.09
Cholestyramine	6	177.5±33.48***	110.9±9.86	23.2±3.99*

<sup>a</sup> Guinea pigs were fed the plain diet or a diet containing either 0.24% w/w cholesterol or 2.5% w/w cholestyramine. The animals fed the plain, cholesterol and cholestyramine diets were killed on days 15, 16 and 14 of the experiment respectively. All animals were killed approximately 4 h after the commencement of the dark period. Slices of liver, ileum and lung were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-<sup>14</sup>C-acetate. Sterol synthesis is expressed as μmoles of 1-<sup>14</sup>C-acetate incorporated into total digitonin-precipitable sterols/g wet tissue/h.

<sup>b</sup> Values are the mean±S.E.

Comparison by Student's t-test with corresponding value for animals receiving plain diet:

\*P<0.05

\*\*\*P<0.001

4 h after the commencement of the dark period.

The effect of feeding the cholesterol and cholestyramine diets on the rates of sterol and fatty acid synthesis in the liver, ileum and lung of the male guinea pigs are shown in Table 13. Cholesterol feeding caused a marked inhibition of sterol synthesis in the liver ( $P < 0.01$ ) and to a lesser extent in the ileum ( $P < 0.05$ ). Sterologogenesis in the lung was also significantly decreased ( $P < 0.05$ ).

Cholestyramine feeding in males also resulted in a 13-fold increase in the rate of hepatic sterologogenesis ( $P < 0.01$ ) but did not significantly affect sterol synthesis in the ileum. In rats, cholestyramine feeding has been reported to markedly stimulate both hepatic (Huff et al., 1963; Gallo et al., 1966; White, 1972) and intestinal (Dietschy, 1968) cholesterologenesis. In the cholestyramine-fed male guinea pigs sterol synthesis in the lung was significantly increased ( $P < 0.01$ ) but to a lesser extent than in females. The rate of fatty acid synthesis in the liver, ileum and lung was not significantly affected by either cholesterol or cholestyramine feeding.

The effects of cholesterol and cholestyramine feeding on the plasma and tissue cholesterol concentrations in both female and male guinea pigs are presented in Table 14. Cholesterol feeding increased the plasma cholesterol concentration but this was significant only in the females ( $P < 0.05$ ). The concentration of cholesterol in the ileum and lung was not significantly affected by cholesterol feeding. Cholestyramine feeding was found to have an extreme hypocholesterolaemic action in both



TABLE 13. Effect of cholesterol and cholestyramine feeding on sterol and fatty acid synthesis in the liver, ileum and lung of male guinea pigs<sup>a</sup>.

Diet	Number of animals	Sterol synthesis			Fatty acid synthesis		
		Liver	Ileum	Lung	Liver	Ileum	Lung
Plain	4	20.7±4.74 <sup>b</sup>	118.1±18.29	16.7±1.11	29.3±5.70	174.1±8.64	346.9±20.32
Cholesterol	4	1.1±0.61**	63.2±4.12*	8.5±2.58*	37.7±6.44	152.6±17.63	232.8±36.65
Cholestyramine	4	270.2±55.34**	98.4±22.05	22.8±0.77**	92.4±28.67	143.8±29.09	304.9±53.00

<sup>a</sup> Guinea pigs were fed the plain diet or a diet containing either 0.24% w/w cholesterol or 2.5% w/w cholestyramine. The animals fed the plain, cholesterol and cholestyramine diets were killed on days 14, 15 and 12 of the experiment respectively. All animals were killed approximately 4 h after the commencement of the dark period. Slices of liver, ileum and lung were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-<sup>14</sup>C-acetate. The data are expressed as μmoles of 1-<sup>14</sup>C-acetate incorporated into sterol (as total digitonin-precipitable sterols) and fatty acids/g wet tissue/h.

<sup>b</sup> Values are the mean±S.E.

Comparison by Student's t-test with corresponding value for animals receiving plain diet:

\*P<0.05

\*\*P<0.01

TABLE 14. Effect of cholesterol and cholestyramine feeding on plasma and tissue cholesterol concentrations in female and male guinea pigs<sup>a</sup>.

Sex	Diet	Number of animals	Days on diet	Plasma cholesterol concentration mg/100ml		Tissue cholesterol concentration mg/g		
				Initial	Final	Liver	Ileum	Lung
Female	Plain	6	15	52.8±7.49 <sup>b</sup>	57.2±5.63	1.8±0.08	1.8±0.08	4.0±0.12
	Cholesterol	6	16	62.7±8.89	125.6±22.72*	3.1±0.34**	1.9±0.11	4.1±0.07
	Cholestyramine	6	14	58.9±7.10	10.9±0.81***	1.6±0.04	1.6±0.08	3.9±0.09
Male	Plain	4	14	58.7±2.83	57.7±3.49	1.7±0.09	1.7±0.05	4.0±0.10
	Cholesterol	4	15	56.1±5.36	78.1±14.3	2.5±0.40	1.8±0.11	4.2±0.29
	Cholestyramine	4	12	56.1±5.29	20.0±1.25***	1.8±0.05	1.5±0.07	3.9±0.22

<sup>a</sup> Female and male guinea pigs were fed the plain diet or a diet containing either 0.24% w/w cholesterol or 2.5% w/w cholestyramine. The concentrations of cholesterol in plasma and various tissues were determined at the end of the experiment.

<sup>b</sup> Values are the mean±S.E. Tissue cholesterol concentrations are expressed on a wet weight basis.

Comparison by Student's t-test with corresponding value for animals receiving plain diet:

\*P<0.05

\*\*P<0.01

\*\*\*P<0.001

females and males. In females, plasma cholesterol concentration was decreased by 81% and in males by 64%. However the tissue cholesterol concentrations were unaffected.

Studies by Horton et al. (1970) and Weis and Dietschy (1975) demonstrated that in rats the degree of stimulation of hepatic cholesterologenesis produced by cholestyramine feeding differed according to whether the animals were killed at the mid-dark or mid-light phase of the lighting cycle. In the first two experiments all guinea pigs were killed at the time in the lighting cycle when hepatic sterologogenesis was maximal. Another experiment with male guinea pigs was therefore carried out in which animals fed the plain or cholestyramine diet were killed 4 h after the commencement of the light period. As shown in Part IV the rate of hepatic sterologogenesis in guinea pigs fed the plain diet was minimal at about this point in the lighting cycle.

The body weights and plasma and tissue cholesterol concentrations of the animals in this study are shown in Table 15. As in the first two experiments the body weights of the animals fed the cholestyramine and plain diets did not differ significantly. The plasma cholesterol concentration was markedly decreased in the cholestyramine-fed animals ( $P < 0.001$ ) but there was no effect on the cholesterol concentration in the liver and ileum. These findings were consistent with those in the experiments in which the animals were killed 4 h after the commencement of the dark period.

In this study sterol synthesis from both



TABLE 15. Effect of cholestyramine feeding on body weights and plasma, liver and ileum cholesterol concentrations in male guinea pigs<sup>a</sup>.

	Diet	
	Plain	Cholestyramine
Number of animals	4	4
Body weight g		
Initial	795±26.1 <sup>b</sup>	769±22.4
Final	849±27.7	794±30.0
Cholesterol concentration		
Plasma mg/100ml		
Initial	71.5±8.66	60.9±4.82
Final	71.4±17.77	20.5±3.46***
Liver mg/g	1.6±0.03	1.5±0.05
Ileum mg/g	1.8±0.07	1.7±0.02

<sup>a</sup> Guinea pigs were fed the plain diet or a diet containing 2.5% w/w cholestyramine for 15 and 14 days respectively. All animals were killed 4 h after the commencement of the light period and cholesterol concentrations in the plasma, liver and ileum determined.

<sup>b</sup> Values are the mean±S.E. Tissue cholesterol concentrations are expressed on a wet weight basis.

Comparison by Student's t-test with initial value:

\*\*\*P<0.001

1- $^{14}\text{C}$ -acetate and 2- $^{14}\text{C}$ -mevalonate was measured in the liver and ileum. As shown in Table 16 cholestyramine feeding caused only a 5-fold increase ( $P < 0.01$ ) in the incorporation of labelled acetate into sterol by the liver in contrast to the 13-fold increase observed in animals killed 12 h earlier in the lighting cycle. The incorporation of labelled acetate into sterol by the ileum was also significantly higher in the cholestyramine-fed animals ( $P < 0.05$ ). However the increase was only marginal. Cholestyramine feeding also resulted in approximately a 2-fold increase in the incorporation of labelled mevalonate into sterol by the liver ( $P < 0.01$ ) but did not significantly affect the incorporation of mevalonate by the ileum.

The effect of fasting on sterol and fatty acid synthesis was studied in two experiments and the data pooled. These studies were carried out with female guinea pigs which had received the plain diet. One group continued to receive the diet ad libitum while another group was fasted for 24 h. All animals were killed approximately 4 h after the commencement of the dark period. As shown in Table 17, fasting for 24 h markedly inhibited sterol synthesis in the liver ( $P < 0.01$ ) and to a lesser extent in the ileum ( $P < 0.001$ ) and lung ( $P < 0.01$ ). However only in the lung did fasting significantly inhibit fatty acid synthesis ( $P < 0.001$ ).

As shown in Table 18, plasma cholesterol concentration was significantly increased by fasting ( $P < 0.05$ ) but the tissue cholesterol concentrations were not affected.

TABLE 16. Effect of cholestyramine feeding on sterol synthesis from 1-<sup>14</sup>C-acetate and 2-<sup>14</sup>C-mevalonate in the liver and ileum of male guinea pigs<sup>a</sup>.

Diet	Number of animals	Sterol synthesis from 1- <sup>14</sup> C-acetate		Sterol synthesis from 2- <sup>14</sup> C-mevalonate	
		Liver	Ileum	Liver	Ileum
Plain	4	10.2±3.31 <sup>b</sup>	92.6±12.10	49.2±3.76	108.6±19.18
Cholestyramine	4	53.0±8.19**	124.2±15.18*	107.4±12.95**	119.7±11.79

<sup>a</sup> Guinea pigs were fed the plain diet or a diet containing 2.5% w/w cholestyramine for 15 and 14 days respectively. All animals were killed 4 h after the commencement of the light period. Slices of liver and ileum were incubated in triplicate in Krebs-Ringer phosphate buffer containing either 1-<sup>14</sup>C-acetate or 2-<sup>14</sup>C-mevalonate. Sterol synthesis is expressed as μmoles of 1-<sup>14</sup>C-acetate or 2-<sup>14</sup>C-mevalonate incorporated into total digitonin-precipitable sterols/g wet tissue/h.

<sup>b</sup> Values are the mean±S.E.

Comparisons by Student's t-test with corresponding value for animals receiving plain diet:

\*P<0.05

\*\*P<0.01



TABLE 17. Effect of fasting on sterol and fatty acid synthesis in the liver, ileum and lung of female guinea pigs<sup>a</sup>.

Treatment	Number of animals	Sterol synthesis			Fatty acid synthesis		
		Liver	Ileum	Lung	Liver	Ileum	Lung
Fed ( <u>ad libitum</u> )	8	19.9±4.31 <sup>b</sup>	121.7±15.37	20.4±2.68	50.1±21.65	113.2±19.47	416.6±42.52
Fasted 24 h	9	4.0±0.93**	43.8±5.86***	9.6±1.12**	8.9±1.95	67.7±12.67	184.8±21.38***

<sup>a</sup> Guinea pigs which had been either fed ad libitum or fasted for 24 h were killed approximately 4 h after the commencement of the dark period. Slices of liver, ileum and lung were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-<sup>14</sup>C-acetate. The data are expressed as  $\mu$ moles of 1-<sup>14</sup>C-acetate incorporated into sterol (as total digitonin-precipitable sterols) and fatty acids/g wet tissue/h.

<sup>b</sup> Values are the mean±S.E.

Comparison by Student's t-test with corresponding value for animals fed ad libitum:

\*\*P<0.01

\*\*\*P<0.001

TABLE 18. Effect of fasting on plasma and tissue cholesterol concentrations in female guinea pigs<sup>a</sup>.

	Treatment	
	Fed ( <u>ad libitum</u> )	Fasted 24 h
Number of animals	8	9
Cholesterol concentration		
Plasma mg/100ml	44.0±7.37 <sup>b</sup>	72.4±6.61*
Liver mg/g	1.5±0.02	1.7±0.08
Ileum mg/g	1.7±0.04	1.8±0.05
Lung mg/g	3.7±0.15	3.5±0.17

<sup>a</sup> Guinea pigs which had been either fed ad libitum or fasted for 24 h were killed and the concentrations of cholesterol in the plasma and tissues determined.

<sup>b</sup> Values are the mean±S.E. Tissue cholesterol concentrations are expressed on a wet weight basis. Comparison by Student's t-test with corresponding value for animals fed ad libitum:

\* P<0.05

#### (b) Discussion.

The studies described in Part IV demonstrated that in guinea pigs the rate of sterologogenesis in the liver was very low and showed a maximum at 3-4 h after the commencement of the dark period. Thus all measurements of sterologogenesis in the present study were made at about this point in the lighting cycle except in one experiment when the animals were killed at about 4 h after the commencement of the light period; the time about which the rate of hepatic sterologogenesis was minimal. In all

animals killed 4 h after the commencement of the dark period, the rate of sterologogenesis in the ileum was approximately 6 times that in the liver while the rates of sterol synthesis in the liver and lung did not differ significantly. These findings confirm those described in Part IV.

The marked inhibition of sterologogenesis in the liver of the males fed cholesterol is consistent with the findings of Swann and Siperstein (1972) who examined the rates of sterologogenesis in various tissues of guinea pigs fed a 5% cholesterol diet for 7 days. The results of their studies which have only recently been published in detail (Swann et al., 1975) show that sterol synthesis in the liver was inhibited by 83%. Sterologogenesis was also markedly inhibited in several other tissues including the ileum and lung, and except in the liver, the inhibition was proportional to the amount of cholesterol accumulated. Furthermore it was shown that only very slight increases in the sterol concentration of the tissues were necessary to inhibit sterologogenesis. Thus although the sterol concentration in the ileum was increased only from 2.2 to 2.5 mg/g, sterologogenesis was inhibited by 73%. In the present study with males a diet containing only 0.24% cholesterol fed for 15 days strongly inhibited sterologogenesis in the ileum and lung although to a lesser extent than in the liver. This inhibition occurred even though the cholesterol concentration in the ileum and lung had not been significantly increased. The failure of cholesterol feeding to inhibit fatty acid synthesis in any of the tissues clearly shows that the low rates of sterol



synthesis were produced by a specific inhibitory effect of cholesterol on sterologogenesis.

In the first experiment the females fed the cholesterol diet showed high individual variation in the rates of hepatic sterologogenesis. Of the 6 females fed the cholesterol diet for 16 days, 4 showed a rate of sterol synthesis in the liver which was less than 0.5  $\mu\text{moles/g/h}$ . However in 1 of the animals the rate was 18.9  $\mu\text{moles/g/h}$ . The plasma and liver cholesterol concentrations in this animal were similar to those in the animals fed the plain diet. However in the 4 animals with very low rates of liver sterol synthesis these concentrations were much higher. Thus in addition to the high individual variation in the rate of hepatic sterologogenesis in guinea pigs receiving a diet containing no added cholesterol (Part IV), there is also marked individual variation between guinea pigs in their ability to metabolise an increased dietary cholesterol intake. Similar variation has been reported in cholesterol-fed baboons (Wilson, 1972a). Although a high cholesterol intake also greatly inhibits liver sterol synthesis in humans (Bhattathiry and Siperstein, 1963) there is insufficient data to indicate whether humans, like guinea pigs and baboons, show high individual variation in their capacity to metabolise an increased dietary cholesterol load and therefore to regulate the rate of sterol production by the liver accordingly.

The effect of cholestyramine feeding on sterol synthesis varied markedly between the three tissues. In the liver and lung sterologogenesis was enhanced, the effect being particularly pronounced in the liver where the rate

of sterol synthesis was increased 5- or 13-fold depending on when the animals were killed in relation to the lighting cycle. In contrast cholestyramine feeding had no effect on sterologogenesis in the ileum in animals killed 4 h after the commencement of the dark period. However in the males fed cholestyramine and killed 4 h after the commencement of the light period, sterol synthesis in the ileum was significantly higher than in animals fed the plain diet and killed at the same time. The difference however was marginal and only a fraction of the increase which cholestyramine feeding produced in liver sterol synthesis. Thus in guinea pigs fed cholestyramine the liver and not the intestine would be quantitatively the most important site of sterologogenesis.

The failure of cholestyramine feeding to significantly enhance sterol synthesis in the ileum at all times throughout the day suggests that intestinal sterologogenesis in the guinea pig is not subject to feedback inhibition by bile acids. In rats cholestyramine feeding enhances cholesterol synthesis in all regions of the small intestine several fold although the effect is not as striking as that produced by biliary diversion (Dietschy, 1968). The role of bile acids in the regulation of intestinal cholesterol synthesis in rats has been extensively studied by Dietschy (1968). In these studies it was shown that there is an inverse relationship between the intraluminal bile acid concentration and the rate of cholesterologenesis in the adjacent intestinal wall. The derepression of intestinal cholesterol synthesis by biliary diversion in rats was prevented by the infusion of whole

bile and of pure bile acids such as taurocholate into the intestinal lumen of bile duct cannulated animals whereas cholestyramine-treated bile did not prevent the enhancement of cholesterol synthesis. These findings together with the consistent observation that cholesterol feeding has relatively little effect on intestinal cholesterogenesis in rats (Dietschy and Siperstein, 1965, 1967; Cayen, 1969), indicate that feedback inhibition by bile acids is almost certainly the major factor in the regulation of intestinal sterologogenesis in this species. A similar feedback mechanism also appears to regulate intestinal sterologogenesis in the squirrel monkey (Dietschy and Wilson, 1968). In humans with biliary obstruction there is a marked increase in intestinal cholesterogenesis indicating the presence of a bile acid negative feedback system (Dietschy and Gamel, 1971). Despite the evidence for a major role of bile acids in directly regulating intestinal sterologogenesis, the possibility that bile acids inhibit intestinal sterol synthesis secondary to some effect on cholesterol metabolism cannot be excluded. Such possible effects include facilitating the movement of cholesterol into the cell, enhancing cholesterol esterification or promoting the formation of some critical cholesterol-lipoprotein complex that may be the actual mediator of feedback (Wilson, 1972b). Recent studies by Shefer et al. (1973a) have provided evidence that dietary sterols as well as bile acids directly regulate the activity of intestinal HMG CoA reductase in rats. They showed firstly that large increases in intestinal bile acid flux failed to reduce enzyme activity when the latter had reached a certain



basal level. Secondly it was shown that the activity of intestinal HMG CoA reductase was markedly reduced when cholesterol and bile acids were fed in combination. This inhibition of enzyme activity appeared not to be a function of intestinal bile acid flux as bile acid secretion rates were nearly identical in rats fed cholesterol plus bile acid and those fed bile acid alone.

The implication of the results of the present study that intestinal sterologogenesis in guinea pigs is not subject to feedback inhibition by bile acids conflicts with the report of Laster et al. (1966) that bile salts markedly inhibited sterol synthesis in guinea pig intestinal mucosa in vitro. In their studies it was found that taurocholate and glycocholate, alone and together strongly inhibited the incorporation of radio-labelled acetate and mevalonate into sterol. In addition the conversion of labelled acetate to  $\text{CO}_2$  was inhibited much less than the incorporation of this precursor into sterol indicating that the effect of the bile salts on sterol biosynthesis was not a consequence of inhibition of uptake of labelled precursor by the mucosa. It was stated that these effects were obtained using purified bile salts. However as these results were reported only in a brief communication the details of how the bile salts were purified are not given. As shown by the studies of Dietschy (1967) the purity of bile salts is of critical importance in determining their effects on intermediary metabolism in vitro. Thus a commercial preparation of taurocholate which had not been further purified was shown to markedly inhibit cholesterol synthesis in rat intestinal

slices. Apart from the non-specific effects which result when unpurified preparations are used, bile salts exert a detergent effect on membrane structures (Dietschy, 1967). Thus it is possible that the apparent specificity of the effect of taurocholate and glycocholate on sterol synthesis in guinea pig intestinal mucosa may only have reflected a greater disruption of those membrane structures associated with sterol biosynthesis than of those concerned with other aspects of cellular function such as the oxidation of acetate. Apart from the general limitations concerning the effects of bile salts in vitro it is surprising that salts of cholic acid would have such strong inhibitory effects on intestinal sterologogenesis in guinea pigs because this bile acid is found only in adult guinea pigs and is present in much smaller amounts than other bile acids (Schoenfield and Sjövall, 1966).

The high sensitivity of intestinal sterologogenesis in the guinea pig to cholesterol feeding shown in these studies and in those by Swann et al. (1975) suggests that direct feedback inhibition by cholesterol may play a major role in the regulation of intestinal sterologogenesis in this species. The finding of Swann et al. (1975) that the injection of Triton WR-1339 into guinea pigs on a low cholesterol diet enhanced sterol synthesis in the ileum approximately 3-fold indicates strong feedback control by endogenous cholesterol. It is possible that the slight stimulatory effect which cholestyramine feeding had on sterologogenesis in the ileum of the male guinea pigs killed during the light period may have resulted from a removal of the inhibitory effect of endogenous cholesterol.

The hypocholesterolaemic effect of cholestyramine in guinea pigs is much greater than in other species. In chickens (Gallo et al., 1966) and humans (Danhof, 1966) cholestyramine lowers plasma cholesterol concentration by less than 25% and has no effect in rats (Huff et al., 1963; Gallo et al., 1966). However in guinea pigs as in chickens and rats (Gallo et al., 1966) cholestyramine feeding does not affect liver cholesterol concentration.

In rats which received a dose of cholestyramine (approximately 1 g/kg bw), the absorption of dietary and endogenous cholesterol was reduced by over 70%, resulting from a decreased concentration of bile acids in the intestinal lumen (Hyun et al., 1963). The faecal excretion of bile acids is markedly enhanced under these conditions (Huff et al., 1963). As plasma cholesterol concentration does not decrease in rats fed cholestyramine, the rate of hepatic and intestinal cholesterologenesis must be increased sufficiently to compensate for the increased faecal loss of bile acids and neutral steroids. In contrast the guinea pigs fed cholestyramine (1.5 g/kg bw/day) in their diet showed an extreme reduction in plasma cholesterol concentration even though the liver produced sterol (principally cholesterol; Ockner and Laster, 1966) at 5-13 times the normal rate. This suggests that the plasma clearance of cholesterol was increased and that the absorption from the intestine of both exogenous and endogenous cholesterol was decreased. An attempt to determine the effect on plasma cholesterol clearance by measuring the activity of cholesterol 7 $\alpha$ -hydroxylase in guinea pigs fed cholestyramine was unsuccessful (Part VI (3)).



The increased excretion of bile acids produced by cholestyramine feeding also results in their decreased intestinal absorption. Thus the enhanced rates of sterologenesis in both the liver and lung in the guinea pigs fed cholestyramine may have resulted because of a release of the inhibition imposed by either bile acids or cholesterol or both, although the relative importance of each cannot be determined from the present study. Recently Swann et al. (1975) reported that the injection of Triton WR-1339 into guinea pigs receiving a low cholesterol diet resulted in great stimulation of hepatic sterologenesis. This suggests that most of the feedback control is probably imposed by endogenous cholesterol although the possibility that bile acids also directly impose some feedback control on hepatic sterol synthesis in the guinea pig cannot be excluded. It has previously been shown that the feeding of bile acids markedly suppresses liver sterol synthesis in this species (Beher et al., 1963). There is evidence from studies with baboons and rats that bile acids may regulate hepatic cholesterol synthesis by a direct effect and not by a secondary effect on cholesterol absorption. Thus Wilson (1972a) showed that in baboons the enhancement of hepatic cholesterol synthesis that followed ileal diversion was not suppressed even to the normal range by a high cholesterol diet, despite the fact that the absorption of dietary cholesterol was unimpaired. Similarly Hamprecht et al. (1971b) demonstrated that feeding cholic acid to rats with diversion of thoracic duct lymph significantly decreased hepatic HMG CoA reductase activity. The depression in enzyme activity was much greater in

animals killed during the dark period than during the light period. Although these findings strongly suggest a direct role of bile acids in regulating hepatic HMG CoA reductase activity, there is other evidence to indicate that bile acids have no such effect. Thus Weis and Dietschy (1969) showed that the enhanced incorporation of acetate into cholesterol by liver homogenates from rats with biliary diversion is not lowered by the intrajejunal or intravenous infusion of taurocholate. However in these studies the rats were killed in the morning. Thus on the basis of the findings of Hamprecht et al. (1971b) the possibility cannot be excluded that Weis and Dietschy may have observed an inhibitory effect of taurocholate had the rats been killed during the dark period. More recent studies by Shefer et al. (1973b) on the effects of feeding sitosterol and bile acids on hepatic HMG CoA reductase activity, have provided further evidence that bile acids may directly regulate the activity of this enzyme.

While there is disagreement concerning the possible role of bile acids in directly regulating cholesterol synthesis in the liver, there is little doubt that hepatic cholesterologenesis is under direct feedback inhibition by cholesterol reaching the liver from the intestine. Recently Nervi and Dietschy (1975) showed that a specific lipoprotein fraction ( $S_f > 8000$ ) in thoracic duct lymph from rats fed a high cholesterol diet produced marked feedback inhibition of hepatic cholesterologenesis when administered as a continuous intravenous infusion to normal rats. A much lower inhibitory effect was produced by serum lipoprotein fractions also prepared from cholesterol-fed rats.

Swann et al. (1975) demonstrated that the injection into guinea pigs of serum lipoproteins from cholesterol-fed chickens resulted in marked inhibition of sterologenesi*s* in the liver, lung and spleen. However similar treatment in rats inhibited sterologenesi*s* only in the liver. These results demonstrate that the feedback sensitivity of sterologenesi*s* in the extrahepatic tissues of the guinea pig is not due to any unique property of guinea pig lipoproteins and, as suggested by Swann and his colleagues, may simply reflect a greater permeability of these tissues to cholesterol-containing lipoproteins.

*As fasting* In the guinea pigs fasted for 24 h marked inhibition of sterologenesi*s* occurred in the liver, ileum and lung although the effect in the liver was greater than in the other tissues. These findings contrast to those reported for the rat and squirrel monkey in which fasting markedly inhibits cholesterol synthesis in the liver but has relatively little effect on cholesterologenesis in the extrahepatic tissues (Dietschy and Siperstein, 1967; Dietschy and Wilson, 1968). Intestinal cholesterologenesis in the human however, is inhibited much more by fasting than it is in the rat and squirrel monkey (Dietschy and Gamel, 1971). It is unlikely that the strong inhibitory effect of fasting on sterologenesi*s* in guinea pig tissues represented only an increased dilution of the labelled acetate by the endogenous acetyl CoA pool, because in liver homogenates prepared from guinea pigs fasted for 40 h, the rate of incorporation of acetate into acetoacetate was not different to that in preparations from fed animals (Sauer, 1960).



The increased plasma cholesterol concentration in the fasted guinea pigs is consistent with the effect observed in rats (Cayen, 1969) and rabbits (Klauda and Zilversmit, 1975). The hypercholesterolaemia in fasted animals probably reflects a decreased efficiency of clearance of plasma  $\beta$ -lipoproteins from the circulation (Klauda and Zilversmit, 1975). Although fasting in rats results in a significant increase in liver cholesterol concentration (Cayen, 1969; Bortz and Steele, 1973) no change was found in the cholesterol concentration in the liver or in the ileum and lung of the fasted guinea pigs. As fasting markedly inhibited sterologogenesis in all three tissues it is certain that the inhibitory effect of fasting is not mediated by changes in cholesterol concentration.

The complexity of the control of sterologogenesis, particularly in the liver, results not only because of the diversity of factors which influence the rate of cholesterol synthesis but also because the effects of these factors can be superimposed. Thus in rats the diurnal rhythm in liver cholesterol synthesis persists in animals in which the rate of cholesterologenesis has been either enhanced by feeding cholestyramine or  $\beta$ -sitosterol or decreased by cholesterol feeding or fasting (Weis and Dietschy, 1975). Although the rate of hepatic sterologogenesis is much lower in guinea pigs than in rats, the control processes are equally as complex. Thus it was found that the degree of stimulation of hepatic sterologogenesis produced by cholestyramine feeding in guinea pigs depended on when the animals were killed in relation to the lighting cycle. A much greater stimulation was found in guinea pigs killed at the peak in the diurnal

rhythm of liver sterol synthesis than in animals killed at the low point of the rhythm. The opposite effect is observed in rats (Horton et al., 1970; Weis and Dietschy, 1975).

The rate-limiting enzyme in the pathway of cholesterol synthesis in rat liver is HMG CoA reductase (Siperstein and Fagan, 1966; Slakey et al., 1972). The changes in the rate of acetyl CoA incorporation into cholesterol which occur during various experimental manipulations such as the feeding of cholesterol or cholestyramine, or fasting, are paralleled exactly by changes in the activity of the reductase (Dietschy and Brown, 1974). The fact that the effects of such manipulations can be superimposed suggests that the final rate of sterol synthesis may be the result of different effectors modifying by different mechanisms the activity of HMG CoA reductase (Weis and Dietschy, 1975). There is as yet no definitive information concerning the identity of these effectors or the mechanisms by which they regulate reductase activity. Before the control of sterologogenesis in guinea pig tissues can be studied at this level it will be necessary to establish which enzyme (or enzymes) is rate-limiting in the pathway of sterologogenesis in the various tissues. Some indication as to the possible sites of control can be obtained from the present studies. It was found that in the liver the incorporation rate of mevalonate into sterol was approximately 3-5 times that of acetate while in the ileum the incorporation rates of the two precursors differed much less. Similar results were obtained in the studies described in Part VI (2) and Part VI (4). However in comparing the rates of

incorporation of mevalonate and acetate into sterol three factors must be considered. Firstly, because of the lack of correction for endogenous dilution, the incorporation rates of acetate do not represent the absolute flux of two-carbon units into sterol. Secondly, although the pH of the incubation medium was optimal for the incorporation of acetate into sterol, it may not have been optimal for the incorporation of mevalonate. Thirdly, for each  $\mu\text{mole}$  of mevalonate incorporated into sterol the equivalent of three two-carbon units are incorporated. On this basis the rate of sterol synthesis from mevalonate would be much higher than from acetate, particularly in the liver. This suggests that in the normal guinea pig the rate-limiting enzyme of sterologogenesis in the liver and intestine is either HMG CoA reductase or an enzyme (or enzymes) catalysing a step before the reduction of HMG CoA. Studies by White (1972) suggest that in rat liver, HMG CoA condensing enzyme may be partially rate-limiting.

Although the rate-limiting enzyme of hepatic sterol synthesis in the guinea pig may occur at or before HMG CoA reductase, it is also likely that an enzyme (or enzymes) subsequent to HMG CoA reductase is under direct feedback control. In the experiment in which male guinea pigs were killed 4 h after the commencement of the light period, the incorporation of mevalonate into sterol by the liver of guinea pigs fed cholestyramine was at least twice that by the liver of guinea pigs fed the plain diet. It is possible that the effect of cholestyramine feeding on mevalonate incorporation would have been much greater than 2-fold had the animals been killed 4 h after



the commencement of the dark period. There was no effect of cholestyramine feeding on mevalonate incorporation into sterol by the ileum. The existence of more than one control point in the pathway of sterol synthesis in the liver and not in the intestine, may explain the greater inhibitory effects of treatments such as cholesterol feeding and fasting on sterologogenesis in the liver than in the intestine. If, as discussed in Part IV, the liver is the major endogenous source of plasma cholesterol in the guinea pig, then the homeostasis of plasma cholesterol concentration would depend greatly on the ability of the liver to rapidly and profoundly alter its rate of sterol production in response to changes in environmental factors such as the cholesterol content of the diet.

The results of the present studies have thus provided an explanation for the low rates of hepatic sterol synthesis relative to the rates of sterologogenesis in the small intestine of the guinea pig. In the normal animal the feedback inhibition produced by both cholesterol and possibly also by bile acids suppresses sterol synthesis in the liver to very low rates compared to those in the small intestine where sterologogenesis is not only less sensitive to the cholesterol negative feedback system than that in the liver, but also is not subject to regulation by the bile acid negative feedback system. Consequently the small intestine alone makes a greater contribution to total body sterol synthesis than does the liver.

PART VI. EFFECTS OF ASCORBIC ACID ON CHOLESTEROL METABOLISM

Evaluation of the method of ascorbic acid supplementation.

There are many factors which affect the ascorbic acid requirement of guinea pigs including the mode of administration (Chatterjee, 1967b). Although 0.5 mg of ascorbic acid/day is sufficient to prevent scurvy (Ginter *et al.*, 1968b), the ascorbic acid requirement of guinea pigs is laid down as 16 mg/kg bw/day (Reid and Bieri, 1972). Thus a system for administering ascorbic acid at this level was established. Administration of ascorbic acid in the drinking water was chosen in preference to other means as it was more convenient and avoided stressing the animals.

The relationship between the water intake and body weight was determined by measuring the intake of water containing 0.010% w/v ascorbic acid of 2 groups of male guinea pigs, each with 4 animals, over 4 consecutive 24-h periods. The mean water intake was  $155 \pm 5.2$  ml/kg bw/day (range 140-170). The mean intake of ascorbic acid was thus 15.5 mg/kg bw/day. The same relationship between water intake and body weight was subsequently observed in animals given either water without added ascorbic acid or water containing 0.200% w/v ascorbic acid. As the administration of water containing 0.010% w/v ascorbic acid allowed an intake approximating the daily requirement, this concentration of ascorbic acid was used as the control level of supplementation in all experiments except one, in which the drinking water contained 0.008% w/v ascorbic acid (Table 19). Although contact of the drinking water with metal surfaces was

avoided, it is possible that some of the ascorbic acid in the drinking water was oxidised by light. However this probably had little effect on the total ascorbic acid intake as it has been shown that when dehydroascorbic acid is administered orally to guinea pigs more than half is reduced to ascorbic acid (Dayton et al., 1966).

The possible contribution of the diet to the ascorbic acid intake of the guinea pigs was calculated from the feed intake, the rate at which ascorbic acid was originally added to the diet (Table 1) and the ascorbic acid content of the individual components of the diet (Friberg and Lohmander, 1970). The feed intake, determined in the experiment in which the water was measured, was  $61 \pm 1.0$  g/kg bw/day (range, 57-66). Thus the maximum amount of ascorbic acid that the guinea pigs would have derived from the diet was 1.5-2 mg/kg bw/day but most of this ascorbic acid was probably degraded during the preparation of the diet (Howarth et al., 1972). Attempts were made to determine the content of ascorbic acid in the diet but the dietary pigments interfered with the assay. Although it was subsequently found that this difficulty could have been overcome by the procedure described by Friberg et al. (1969), further analysis was not attempted because the guinea pigs were found to develop scurvy when fed the plain diet without ascorbic acid supplementation. The results of this study are described in Part VI (1). As shown by the various experiments described in Part VI, there was a strong correlation between the concentrations of ascorbic acid in the tissues of guinea pigs and the level of supplementation given in the drinking water.



Thus the animals must have derived negligible amounts of ascorbic acid from the diet.

One of the overriding advantages of the guinea pig as a model for studies on the effects of ascorbic acid on cholesterol metabolism is that the half-life of ascorbic acid in this species is about 4 days (Burns *et al.*, 1956; Ginter *et al.*, 1971b). Thus the tissues can be rapidly depleted of ascorbic acid. It is generally found that during the first two weeks of feeding guinea pigs a vitamin C-free diet, the animals consume normal amounts of feed and show normal weight gains. However during the third week the various symptoms of scurvy become evident. By the fourth week the animals are severely scorbutic and usually die during this period (Gasterling and Long, 1951; Ginter *et al.*, 1955c, 1958b). The intake of ascorbic acid required to prevent acute scurvy in guinea pigs has been shown to be as little as 0.5 mg/day (Ginter *et al.*, 1958b) but work from other laboratories has indicated a higher requirement (Chatterjee, 1967b). Thus as it was intended to examine the effects of dietary vitamin C deficiency on various aspects of cholesterol metabolism, in guinea pigs it was necessary to establish whether the plain diet, when fed without ascorbic acid supplementation, was scorbutogenic. Although vitamin C had been originally incorporated into the diet, it seemed likely that this, as well as the vitamin C present in various components of the diet, would have been degraded in the preparation and storage of the diet. Attempts to determine the ascorbic acid content of the diet by direct assay were unsuccessful because of interference by dietary pigments.

PART VI (1). Effects of severe ascorbic acid deficiency on cholesterol metabolism in guinea pigs.

One of the over-riding advantages of the guinea pig as a model for studies on the effects of ascorbic acid on cholesterol metabolism is that the half-life of ascorbic acid in this species is about 4 days (Burns et al., 1956; Ginter et al., 1971b). Thus the tissues can be rapidly depleted of ascorbic acid. It is generally found that during the first two weeks of feeding guinea pigs a vitamin C-free diet, the animals consume normal amounts of feed and show normal weight gains. However during the third week the various symptoms of scurvy become evident. By the fourth week the animals are severely scorbutic and usually die during this period (Oesterling and Long, 1951; Ginter et al., 1965c, 1968b). The intake of ascorbic acid required to prevent acute scurvy in guinea pigs has been shown to be as little as 0.5 mg/day (Ginter et al., 1968b) but work from other laboratories has indicated a higher requirement (Chatterjee, 1967b). Thus as it was intended to examine the effects of dietary vitamin C deficiency on various aspects of cholesterol metabolism in guinea pigs it was necessary to establish whether the plain diet, when fed without ascorbic acid supplementation, was scorbutogenic. Although vitamin C had been originally incorporated into the diet, it seemed likely that this, as well as the vitamin C present in various components of the diet, would have been degraded in the preparation and storage of the diet. Attempts to determine the ascorbic acid content of the diet by direct assay were unsuccessful because of interference by dietary pigments.

The present study therefore examined the effect of feeding the plain diet without ascorbic acid supplementation on the survival of male and female guinea pigs. The effect on plasma and tissue cholesterol and ascorbic acid concentrations was also examined.

(a) Results.

As there was a shortage of guinea pigs at the time this experiment was carried out, no control groups specific to the experiment were included. However at about the same time, a preliminary study on the effect of cholesterol feeding on the cholesterolaemic response of guinea pigs was being carried out. This study included as controls, male and female guinea pigs fed the plain diet with normal levels of ascorbic acid supplementation in their drinking water. These animals were of similar age to those which were unsupplemented. Thus the data collected from them on body weight gain, feed intake and plasma cholesterol concentration were used for comparison with that from the animals receiving the plain diet without supplementation. However as the experiments with the two groups of animals were terminated after widely differing periods, data on the tissue concentrations of cholesterol and ascorbic acid in normal animals were taken from other experiments for comparison with that from the unsupplemented animals.

The effect of feeding the plain diet without and with ascorbic acid supplementation on the body weights of both male and female guinea pigs is shown in Figure 17. The unsupplemented animals gained weight during the first 12 days but thereafter showed a marked decrease in weight.



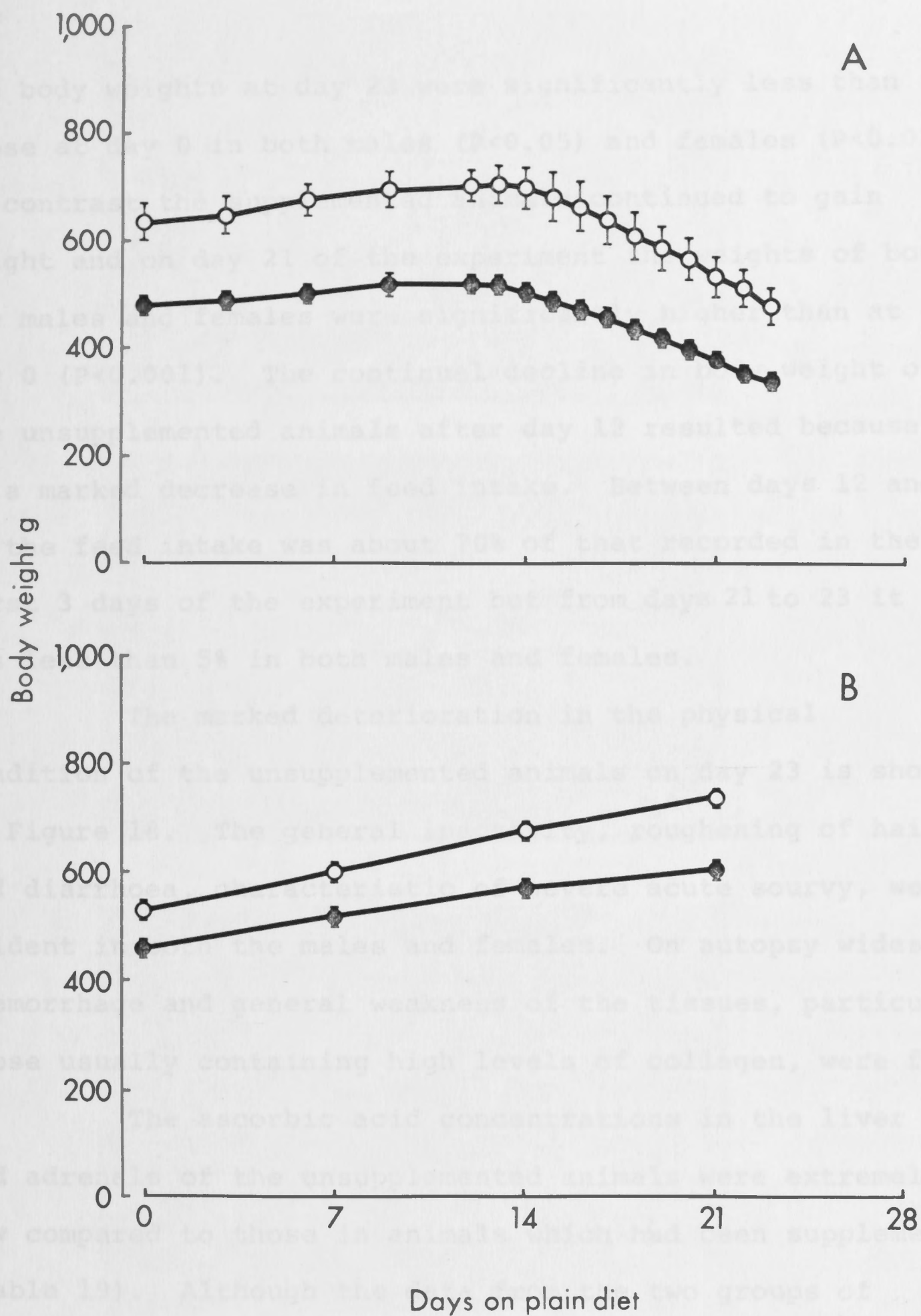


Figure 17. Body weights of male and female guinea pigs fed the plain diet without and with ascorbic acid supplementation. Body weights of male (○—○) and female (●—●) guinea pigs fed the plain diet without (A) and with (B) ascorbic acid supplementation were recorded at the intervals indicated. In the study with the unsupplemented animals there were 5 males and 3 females and in that with the supplemented animals there were 5 males and 5 females. Both studies were commenced only 4 days after the animals had been transferred to the light-controlled room. Values are the mean±S.E.

The body weights at day 23 were significantly less than those at day 0 in both males ( $P < 0.05$ ) and females ( $P < 0.01$ ). In contrast the supplemented animals continued to gain weight and on day 21 of the experiment the weights of both the males and females were significantly higher than at day 0 ( $P < 0.001$ ). The continual decline in body weight of the unsupplemented animals after day 12 resulted because of a marked decrease in feed intake. Between days 12 and 15 the feed intake was about 70% of that recorded in the first 3 days of the experiment but from days 21 to 23 it was less than 5% in both males and females.

The marked deterioration in the physical condition of the unsupplemented animals on day 23 is shown in Figure 18. The general inactivity, roughening of hair and diarrhoea, characteristic of severe acute scurvy, were evident in both the males and females. On autopsy widespread haemorrhage and general weakness of the tissues, particularly those usually containing high levels of collagen, were found.

The ascorbic acid concentrations in the liver and adrenals of the unsupplemented animals were extremely low compared to those in animals which had been supplemented (Table 19). Although the data from the two groups of animals cannot be validly compared statistically, it is clear that the tissues of the unsupplemented animals were almost entirely depleted of ascorbic acid. These tissue ascorbic acid concentrations are similar to those reported by Ginter et al. (1967, 1968b) and demonstrate that the guinea pigs derived negligible amounts of ascorbic acid from the diet.



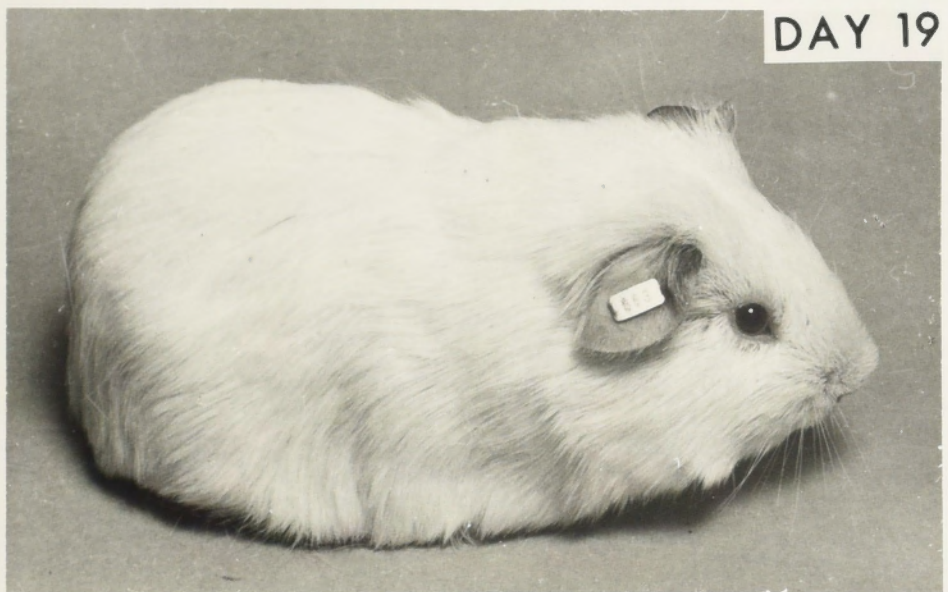
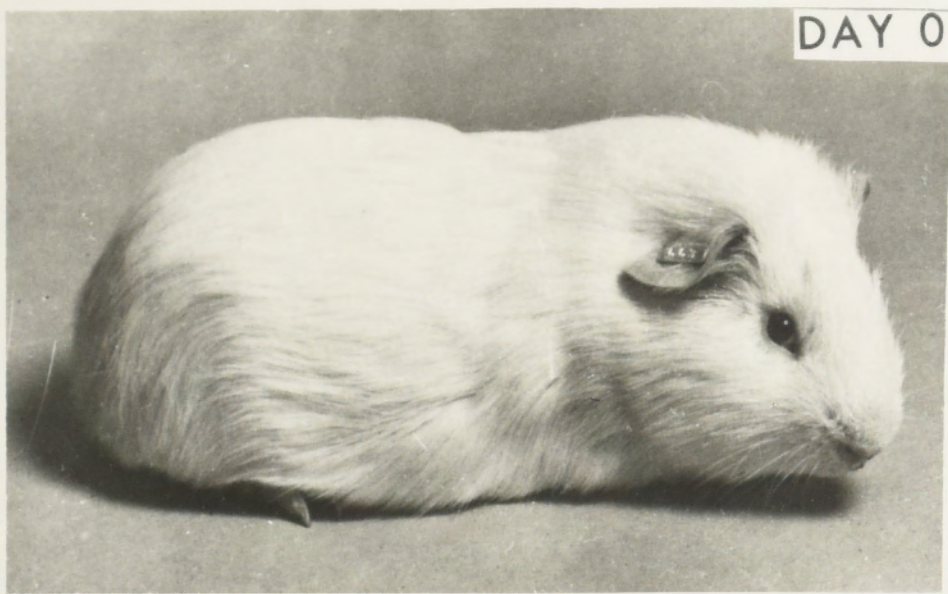


Figure 18. *Male guinea pig at various stages of acute scurvy.* This animal was maintained on the plain diet for 23 days without ascorbic acid supplementation. The photograph on day 23 shows the marked deterioration in physical condition associated with late acute scurvy and is representative of the effect observed in all animals maintained on the plain diet without ascorbic acid supplementation.



TABLE 19. Ascorbic acid concentrations in the tissues of guinea pigs with severe ascorbic acid deficiency.

Treatment	Number of Animals	Ascorbic acid concentration mg/100g	
		Liver	Adrenal
Scorbutic <sup>a</sup>			
Male	5	1.6±0.24 <sup>c</sup>	5.5±0.72
Female	3	1.5±0.02	4.3±1.29
Supplemented <sup>b</sup>			
Male	18	21.4±1.75	92.0±8.60
		(range 10.3-35.6)	(range 38.3-134.8)

<sup>a</sup> Animals were fed the plain diet for 23 days without ascorbic acid supplementation.

<sup>b</sup> Male guinea pigs were fed the plain diet and supplemented with ascorbic acid (0.008% w/v in drinking water). This study was completed several months before the experiment on severe ascorbic acid deficiency was carried out.

<sup>c</sup> Values are expressed on a wet weight basis and are the mean±S.E.

As shown in Figure 19 the plasma cholesterol concentration in the unsupplemented animals increased greatly between day 10 and day 17 (males,  $P < 0.001$ ; females,  $P < 0.01$ ). Although there was a decrease between day 17 and day 23 this was not significant. In the experiment with the supplemented animals plasma cholesterol concentration was determined at intervals different to those with the unsupplemented animals. On day 21 the plasma cholesterol concentration was significantly higher than at day 0 in both the males ( $P < 0.01$ ) and females ( $P < 0.05$ ). However the

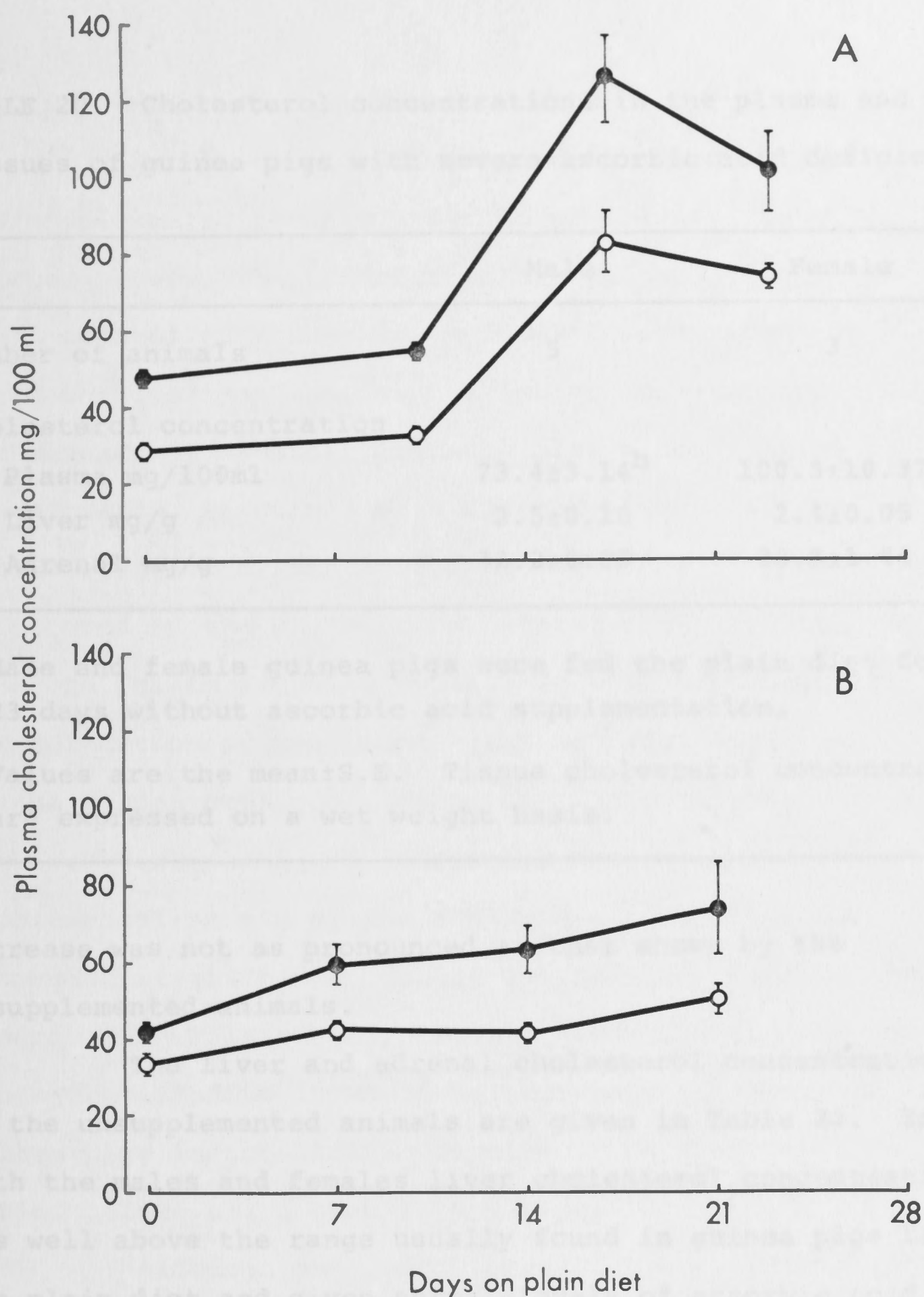


Figure 19. Plasma cholesterol concentration in male and female guinea pigs fed the plain diet without and with ascorbic acid supplementation. Plasma cholesterol concentration in male (○—○) and female (●—●) guinea pigs fed the plain diet without (A) and with (B) ascorbic acid supplementation was determined at the intervals indicated. In the study with the unsupplemented animals there were 5 males and 3 females and in that with the supplemented animals there were 5 males and 5 females. Both studies were commenced only 4 days after the animals had been transferred to the light-controlled room. Values are the mean±S.E.

TABLE 20. Cholesterol concentrations in the plasma and tissues of guinea pigs with severe ascorbic acid deficiency<sup>a</sup>.

	Male	Female
Number of animals	5	3
Cholesterol concentration		
Plasma mg/100ml	73.4±3.14 <sup>b</sup>	100.5±10.37
Liver mg/g	2.5±0.10	2.4±0.05
Adrenal mg/g	32.2±6.88	20.8±1.44

<sup>a</sup> Male and female guinea pigs were fed the plain diet for 23 days without ascorbic acid supplementation.

<sup>b</sup> Values are the mean±S.E. Tissue cholesterol concentrations are expressed on a wet weight basis.

increase was not as pronounced as that shown by the unsupplemented animals.

The liver and adrenal cholesterol concentrations in the unsupplemented animals are given in Table 20. In both the males and females liver cholesterol concentration was well above the range usually found in guinea pigs fed the plain diet and given normal levels of ascorbic acid supplementation (1.5-1.8 mg/g). In contrast adrenal cholesterol concentration was below the range usually found in normal animals (40-65 mg/g). Ginter et al. (1965a) reported that liver cholesterol concentration was unaffected by acute scurvy in guinea pigs. However in the adrenal, cholesterol concentration significantly increased in the early stage of the condition but decreased to less than normal in the late stage of acute scurvy.



(b) Discussion.

In the experiments with both the unsupplemented and supplemented animals a period of only 4 days was allowed between the transfer of the guinea pigs to the light-controlled room and the initial measurement of plasma cholesterol concentration. A longer adjustment period was not considered necessary because plasma cholesterol concentration in guinea pigs did not show a diurnal variation (Part I) and therefore would not have been influenced by the change from natural lighting conditions to a controlled lighting cycle. It was found however that the relocation of the guinea pigs markedly affected their plasma cholesterol concentration. In both the males and females which continued to receive ascorbic acid supplementation the plasma cholesterol concentration increased significantly during the first 3 weeks of the experiment. This clearly demonstrated that in all subsequent studies it would be necessary to maintain the guinea pigs in the light-controlled room for about this period before being used in any experiment.

Although the unsupplemented guinea pigs showed a greater and more rapid increase in plasma cholesterol concentration than those which were supplemented, it is difficult to be certain that this was the specific result of the withdrawal of ascorbic acid supplementation. This is because in subsequent studies in which the guinea pigs had been maintained in the light-controlled room for at least 3 weeks, it was found that 15 days after the withdrawal of ascorbic acid supplementation the plasma cholesterol concentration had not been affected significantly

(Part VI (2), Part VI (3)). Although other studies have reported a significant increase in plasma cholesterol concentration in severely scorbutic guinea pigs (Bolker et al., 1956; Lahiri and Banerjee, 1956; Banerjee and Ghosh, 1960), Ginter et al. (1965a) demonstrated a significant decrease in plasma cholesterol concentration by day 18 of acute vitamin C deficiency and a return to almost normal concentration by day 25 of the condition. Ginter and his colleagues attributed the increase in plasma cholesterol in the terminal phase of acute scurvy to the secondary effect of fasting which is generally found to result in hypercholesterolaemia (Part V).

The effect of severe scurvy on organ weight could not be assessed in the present study because of the lack of data on organ weights in normal animals which were of similar body weight to those with scurvy. However it has generally been found that severe vitamin C deficiency in guinea pigs results in a decrease in liver weight and an increase in adrenal weight (Chatterjee, 1967a). When such effects are taken into account it probably means that, although the liver cholesterol concentration in the scorbutic animals was well above the normal range, the total liver cholesterol content may not have differed from normal. Similarly the total cholesterol content of the adrenals may not have differed from normal.

In male guinea pigs with chronic latent scurvy the plasma and liver cholesterol concentrations are consistently increased (Ginter and Nemec, 1969; Ginter et al., 1969d, 1971a, 1972b, 1973b; Ginter, 1973; Nambisan and Kurup, 1975) although organ weight is not affected

(Ginter et al., 1968b). Thus, while the use of this model best simulates the condition prevalent in human populations, there is some disadvantage in that it usually takes several months to produce this condition in guinea pigs. Alternatively guinea pigs with severe vitamin C deficiency are unsuitable because of the secondary effects on cholesterol metabolism which cannot be corrected for by pair feeding. It was thus decided to study the effect of dietary vitamin C deficiency on sterol synthesis and cholesterol 7 $\alpha$ -hydroxylase using guinea pigs in the early stage of acute scurvy.

deficiency on intestinal sterogenesis.

A recent study using liver homogenates from baboons with latent scurvy demonstrated that the incorporation of labelled mevalonate into cholesterol was decreased to a greater extent than was the incorporation of labelled acetate (Weight et al., 1974). This implied that ascorbic acid is involved in the regulation of an enzyme subsequent to  $\beta$ -hydroxy- $\beta$ -methylglutaryl Coenzyme A (HMG CoA) reductase. It therefore seemed important to examine this possibility in another species. In the present study then, the effect of acute ascorbic acid deficiency in the early stage on hepatic and intestinal sterogenesis in guinea pigs has been examined using both labelled acetate and mevalonate as precursors.

#### (a) Results.

The unsupplemented or scorbutic animals were killed on the 15th day of the experiment and the supplemented or control group on the 16th day. On the 14th day the feed intake of the unsupplemented group was



PART VI (2). Effect of acute ascorbic acid deficiency on sterologogenesis in the liver and ileum of guinea pigs.

In severely scorbutic guinea pigs sterol synthesis in various tissues has been reported to increase (Becker *et al.*, 1953; Guchhait and Ganguli, 1961a) or to show no change (Bolker *et al.*, 1956). In guinea pigs with chronic latent scurvy sterol synthesis in the liver has been shown not to be affected (Ginter and Nemec, 1969). In all of these studies labelled acetate has been used as the precursor and none has examined the effect of ascorbic acid deficiency on intestinal sterologogenesis.

A recent study using liver homogenates from baboons with latent scurvy demonstrated that the incorporation of labelled mevalonate into cholesterol was decreased to a greater extent than was the incorporation of labelled acetate (Weight *et al.*, 1974). This implied that ascorbic acid is involved in the regulation of an enzyme subsequent to  $\beta$ -hydroxy- $\beta$ -methylglutaryl Coenzyme A (HMG CoA) reductase. It therefore seemed important to examine this possibility in another species. In the present study then, the effect of acute ascorbic acid deficiency in the early stage on hepatic and intestinal sterologogenesis in guinea pigs has been examined using both labelled acetate and mevalonate as precursors.

(a) Results.

The unsupplemented or scorbutic animals were killed on the 15th day of the experiment and the supplemented or control group on the 16th day. On the 14th day the feed intake of the unsupplemented group was

about 80% of that of the supplemented group. This may account for the slightly higher body weight gain of the supplemented animals (Table 21). As also shown in Table 21 the liver weight in the scorbutic group was significantly less than in the supplemented group ( $P < 0.05$ ). However, this largely reflected the difference in body weight as the liver weight, expressed on a body weight basis, did not differ significantly between the scorbutic and supplemented animals. Adrenal weight did not differ significantly between the two groups (Table 21).

The ascorbic acid concentrations in the blood and tissues of the unsupplemented animals were significantly lower (whole blood,  $P < 0.01$ ; tissues,  $P < 0.001$ ) than those in the supplemented animals (Table 21). However as expected the concentrations at this stage of the deficiency were higher than those in the guinea pigs which had been unsupplemented for 23 days (Table 19).

Despite the significant decrease in ascorbic acid concentrations, there was no change in cholesterol concentrations in the plasma and tissues except in the liver which showed a significantly higher concentration in the unsupplemented animals ( $P < 0.05$ ) (Table 21). However the difference was only marginal and the liver cholesterol concentration in the scorbutic group was within the range observed in normal guinea pigs in other studies. The lack of an effect on plasma cholesterol concentration contrasts to the results of the experiment described in Part VI (1) in which a marked increase in plasma cholesterol concentration was found in guinea pigs after 17 days of feeding the plain diet without ascorbic acid supplementation.

TABLE 21. Effect of acute ascorbic acid deficiency on body weights, organ weights and cholesterol and ascorbic acid concentrations in the plasma and tissues of male guinea pigs<sup>a</sup>.

	Supplemented	Unsupplemented
Number of animals	4	5
Body weight g		
Initial	800±11.8 <sup>b</sup>	791±28.6
Final	855±19.3	804±32.6
Organ weight		
Liver g	42.7±1.51	36.9±1.70* <sup>c</sup>
Adrenals mg	370±11.5	299±36.1
Cholesterol concentration		
Plasma mg/100ml		
Initial	60.5±6.49	56.3±6.24
Final	51.4±3.23	43.8±2.62
Liver mg/g	1.6±0.04	1.7±0.02*
Ileum mg/g	1.8±0.05	1.8±0.03
Adrenal mg/g	49.6±1.97	43.9±4.16
Ascorbic acid concentration		
Whole blood mg/100ml	1.19±0.139	0.57±0.041**
Liver mg/100g	30.8±1.72	11.3±0.52***
Ileum mg/100g	24.8±2.16	7.0±0.45***
Adrenal mg/100g	66.8±3.16	13.2±0.62***

<sup>a</sup> Two groups of male guinea pigs were fed the plain diet. One group received supplementary ascorbic acid in their drinking water (0.010% w/v) while the other group was not supplemented. The unsupplemented and supplemented groups were killed on days 15 and 16 of the experiment respectively.

<sup>b</sup> Values are the mean±S.E. Organ weights and cholesterol and ascorbic acid concentrations are expressed on a wet weight basis.

<sup>c</sup> Comparison by Student's t-test with corresponding value for supplemented animals:

\*P<0.05

\*\*P<0.01

\*\*\*P<0.001



However unlike the present study in which the guinea pigs were maintained in the light-controlled room for several weeks before the withdrawal of vitamin C supplementation, those in the preceding study were maintained for only 4 days before being deprived of vitamin C supplementation. Thus, in agreement with Ginter et al. (1965a) the present study has shown that acute ascorbic acid deficiency in the early stage in guinea pigs does not lead to enhanced plasma and liver cholesterol concentrations. These appear to occur only after a prolonged dietary insufficiency of vitamin C (Ginter and Nemec, 1969; Ginter et al., 1969d, 1971a, 1972b, 1973b; Ginter, 1973; Nambisan and Kurup, 1975).

The rates of hepatic and intestinal sterologogenesis are given in Table 22. The incorporation of both labelled acetate and mevalonate into sterol in the livers of the scorbutic guinea pigs did not differ significantly from that in the controls. However in the ileum, sterol synthesis from both acetate and mevalonate was less in the scorbutic group than in the controls, the difference in mevalonate incorporation being significant ( $P < 0.05$ ). The decrease in acetate incorporation almost attained statistical significance ( $P = 0.055$ ).

Although not related to the study of the effect of vitamin C deficiency on sterologogenesis, an additional experiment was carried out at the same time to determine whether there was any difference in the ascorbic acid status of the guinea pigs supplemented with natural sources of vitamin C and those adapted to receiving their daily vitamin C requirement in their drinking water. As shown

TABLE 22. Effect of acute ascorbic acid deficiency on sterol synthesis in the liver and ileum of male guinea pigs<sup>a</sup>.

Treatment	Number of animals	Sterol synthesis from 1- <sup>14</sup> C-acetate μmoles incorporated/g tissue/h <sup>b</sup>		Sterol synthesis from 2- <sup>14</sup> C-mevalonate μmoles incorporated/g tissue/h	
		Liver	Ileum	Liver	Ileum
Supplemented	4	15.8±6.75 <sup>c</sup>	118.5±9.78	52.5±9.74	132.4±9.75
Unsupplemented	5	16.7±4.22	95.5±4.62	56.3±7.27	104.2±4.63*

<sup>a</sup> See footnote a of Table 21.

<sup>b</sup> Slices of liver and ileum were incubated in triplicate in Krebs-Ringer phosphate buffer containing either 1-<sup>14</sup>C-acetate or 2-<sup>14</sup>C-mevalonate.

<sup>c</sup> Values are the mean±S.E. Comparison by Student's t-test with corresponding value for supplemented animals:

\*P<0.05

in Table 23 the ascorbic acid concentrations in the liver and adrenals were similar in animals which received the different treatments. The significantly higher blood ascorbic acid concentration in the animals given vitamin C in their drinking water ( $P < 0.05$ ) may have reflected the difference in the mode of administration rather than a difference in absolute intake. The similarity in tissue ascorbic acid concentrations indicated that the guinea pigs supplemented with natural sources of ascorbic acid received about the same level of vitamin C as those given 0.010% w/v ascorbic acid in their drinking water.

The body and organ weights and the plasma and tissue cholesterol concentrations of the guinea pigs given natural sources of vitamin C are also shown in Table 23. Although the body weight of these animals was similar to the final weight of the supplemented group (Table 21), the liver and adrenal weight of the former group were significantly less (liver,  $P < 0.01$ ; adrenal,  $P < 0.05$ ). The explanation of this difference is obscure. As expected, the plasma cholesterol concentration of the supplemented animals in the light-controlled room was significantly higher than in the animals maintained in the room where they had been bred (Table 23). Although the latter group showed a significantly higher liver cholesterol concentration ( $P < 0.01$ ) the difference between the groups was only marginal. Furthermore the liver cholesterol concentration of the group given natural sources of vitamin C was within the range observed in normal guinea pigs maintained in the light-controlled room. There was no significant difference in adrenal cholesterol concentration between the two groups.



TABLE 23. Body and organ weights and cholesterol and ascorbic acid concentrations in the blood and tissues of male guinea pigs fed the plain diet supplemented with natural sources of ascorbic acid<sup>a</sup>.

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Body weight g	829±37.1 <sup>b</sup>
Organ weight	
Liver g	32.1±1.46** <sup>c</sup>
Adrenals mg	326±11.1*
Cholesterol concentration	
Plasma mg/100ml	37.0±2.69*
Liver mg/g	1.8±0.01**
Adrenal mg/g	53.7±2.44
Ascorbic acid concentration	
Whole blood mg/100ml	0.74±0.064*
Liver mg/100g	31.0±1.44
Adrenal mg/100g	75.3±4.93

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<sup>a</sup> At about the same time as the experiment on the effect of acute ascorbic acid deficiency on sterologogenesis was carried out, 4 male guinea pigs were taken directly from the room where they had been bred, and killed. These animals were the same age as those used in the experiment on the effect of acute scurvy on sterologogenesis, but were fed the plain diet supplemented with pumpkin and freshly cut fodder.

<sup>b</sup> Values are the mean±S.E. Organ weights and tissue cholesterol and ascorbic acid concentrations are expressed on a wet weight basis.

<sup>c</sup> Comparison by Student's t-test with corresponding value for animals in supplemented group in Table 21:

\*P<0.05      \*\*P<0.01

(b) Discussion.

The aim of the present study was to examine hepatic and intestinal sterologogenesis in guinea pigs at the stage of acute ascorbic acid deficiency in which the tissue levels of ascorbic acid had been significantly decreased but before feed intake and body weight gain had been markedly affected. It was of particular interest to examine intestinal sterologogenesis in the scorbutic guinea pig because this had not previously been studied and, more particularly, because it had been shown that in the guinea pig the rate of intestinal sterologogenesis greatly exceeded that in the liver (Part IV). It was considered that any effect of ascorbic acid deficiency on sterologogenesis might therefore be more readily detected in the intestine than in the liver.

The ascorbic acid concentrations in the liver and ileum of the unsupplemented guinea pigs were 37% and 28% respectively of those in the controls. While the tissue ascorbic acid concentrations of the scorbutic groups were higher than those reported for guinea pigs with chronic latent scurvy (Ginter et al., 1968b), the decrease in the ascorbic acid concentration of the ileum was sufficient to lower the rate of sterol synthesis. Although this decrease was not significant with respect to the incorporation of labelled acetate it was significant for the incorporation of labelled mevalonate. At the time the scorbutic animals were killed their feed intake was about 80% of that of the supplemented animals. It is unlikely that this caused the decreased rate of intestinal sterologogenesis because hepatic sterologogenesis in the guinea

pig is more sensitive to decreased caloric intake than is that in the intestine (Part V). The rates of hepatic sterologogenesis in the scorbutic and supplemented groups were similar.

The lack of an effect of vitamin C deficiency on hepatic sterol synthesis may have resulted because the liver ascorbic acid concentration had not decreased to a sufficiently critical level. However Ginter and Nemec (1969) had earlier found that the incorporation of acetate into cholesterol in the liver in vivo did not differ significantly between guinea pigs with chronic latent scurvy and those which had received adequate ascorbic acid supplementation. In contrast to these findings Weight et al. (1974) showed that in liver homogenates prepared from baboons with latent scurvy, the synthesis of cholesterol from both labelled acetate and mevalonate was significantly decreased. The effect on mevalonate incorporation was much greater than on acetate incorporation.

The addition of ascorbic acid to homogenates prepared from livers of either scorbutic or normal baboons has been shown to have no effect on the incorporation of either acetate or mevalonate into cholesterol (Weight et al., 1974). Preliminary studies with liver slices from scorbutic and normal guinea pigs, carried out before the present work, showed that the incorporation of acetate into sterol was not affected by the addition of ascorbic acid to the incubation medium. These studies have not been further pursued. As described in Part VI (4), the saturation of guinea pig tissues with ascorbic acid by the prolonged intake of high levels of vitamin C had no



effect on the incorporation of labelled acetate or mevalonate into sterol by the ileum or liver. It thus appears that while sterol synthesis is probably decreased in ascorbic acid deficiency it cannot be increased above normal rates by an intake of ascorbic acid above the normal requirement.

Weight et al. (1974) have concluded that ascorbic acid is involved in the regulation of an enzyme in the biosynthetic pathway of cholesterol subsequent to the rate-limiting enzyme HMG CoA reductase. The findings of the present study support this conclusion. Popják et al. (1958) showed that ascorbic acid could act as a cofactor in the conversion of labelled mevalonate to sterol by rat liver microsomes. Novitskii (1971) has made reference to the finding by other workers that squalene synthetase is decreased in ascorbic acid deficiency but unfortunately the details of this work are not available. The measurement of enzymes such as HMG CoA reductase and squalene synthetase in scorbutic and resupplemented animals should establish more clearly the nature of the role of ascorbic acid in regulating cholesterol synthesis.

As discussed in Part IV it seems probable that although the intestine in the guinea pig is the major site of sterol synthesis, its contribution to the plasma cholesterol pool may be very small because only a fraction of the sterol synthesised in the intestine is cholesterol. Thus, any change in the rate of intestinal sterologogenesis in the guinea pig produced by dietary ascorbic acid deficiency, would be expected to have little effect on the plasma cholesterol concentration. In man however, a decrease in intestinal sterologogenesis of the extent

observed in the scorbutic guinea pigs in the present study, may lead to a reduction in plasma cholesterol concentration. This is because the principal sterol produced by the human intestine is cholesterol (Dietschy and Gamel, 1971) and it is believed that in man, cholesterol synthesised in the extrahepatic tissues is a major source of plasma cholesterol (Cox et al., 1963). Thus if chronic latent scurvy in humans also reduces cholesterol absorption as it does in guinea pigs (Ginter et al., 1969c), then the condition must lead to a decrease in the amount of cholesterol entering the body pools. However as chronic latent scurvy results in enhanced plasma and liver cholesterol concentrations, any reduction in the entry of cholesterol into the body pools must be more than counteracted by a decrease in the output of cholesterol resulting from the impairment of bile acid synthesis. guinea pigs (Leber et al., 1970; Deckwitz et al., 1973) and this effect is reversed by repletion with ascorbic acid. Ginter and Nemes (1972) have shown that the time course of the enhancement of the oxidation of 26- $^{14}$ C-cholesterol to  $^{14}$ CO $_2$  produced by injecting ascorbic acid into chronically scorbutic guinea pigs closely parallels the increase in the concentration of cytochrome P-450 observed under these conditions by Leber et al. (1970). Secondly Ginter (1975) has demonstrated that there is no difference in the conversion of 26- $^{14}$ C-7 $\alpha$ -hydroxycholesterol to  $^{14}$ CO $_2$  between guinea pigs with chronic ascorbic acid deficiency and normal animals. This evidence strongly suggests that ascorbic acid regulates bile acid synthesis at the stage of 7 $\alpha$ -hydroxylation of cholesterol and not in subsequent

PART VI (3). Effects of acute ascorbic acid deficiency and cholestyramine feeding on cholesterol 7 $\alpha$ -hydroxylase in guinea pigs.

There is substantial evidence to show that in scorbutic guinea pigs bile acid synthesis and excretion are significantly reduced (Guchhait et al., 1963; Ginter et al., 1971a, 1972b, 1973b; Ginter, 1973, 1975). Although Ginter and his colleagues believe that ascorbic acid controls bile acid synthesis by regulation of the activity of the rate-limiting enzyme in the pathway, cholesterol 7 $\alpha$ -hydroxylase, this is so far supported by only two lines of indirect evidence. Firstly, the concentration of cytochrome P-450 which is involved in the 7 $\alpha$ -hydroxylation of cholesterol (Wada et al., 1969), is substantially reduced in scorbutic guinea pigs (Leber et al., 1970; Degkwitz et al., 1973) and this effect is reversed by repletion with ascorbic acid. Ginter and Nemec (1972) have shown that the time course of the enhancement of the oxidation of 26-<sup>14</sup>C-cholesterol to <sup>14</sup>CO<sub>2</sub> produced by injecting ascorbic acid into chronically scorbutic guinea pigs closely parallels the increase in the concentration of cytochrome P-450 observed under these conditions by Leber et al. (1970). Secondly Ginter (1975) has demonstrated that there is no difference in the conversion of 26-<sup>14</sup>C-7 $\alpha$ -hydroxycholesterol to <sup>14</sup>CO<sub>2</sub> between guinea pigs with chronic ascorbic acid deficiency and normal animals. This evidence strongly suggests that ascorbic acid regulates bile acid synthesis at the stage of 7 $\alpha$ -hydroxylation of cholesterol and not in subsequent



reactions. However there is no data available concerning the effect of dietary ascorbic acid deficiency on the activity of cholesterol 7 $\alpha$ -hydroxylase.

Thus in the present study an attempt has been made to measure the activity of cholesterol 7 $\alpha$ -hydroxylase in guinea pigs in the early stage of acute ascorbic acid deficiency. As there had been difficulty in detecting enzyme activity in preliminary experiments this study also included the measurement of enzyme activity in a group of guinea pigs fed cholestyramine. In rats fed cholestyramine cholesterol 7 $\alpha$ -hydroxylase activity is increased several fold (Shefer et al., 1968; Boyd et al., 1973; Mitropoulos et al., 1973).

(a) Results.

In these studies the control animals were those fed the plain diet with ascorbic acid supplementation while the scorbutic animals were those fed the plain diet without supplementation. A third group was fed the cholestyramine diet and received the same level of ascorbic acid supplementation as the controls. The group fed the cholestyramine diet and 2 control animals were killed on day 14 of the experiment while the scorbutic group and the other 2 control animals were killed on day 15. On day 13 the feed consumption of all three groups was within the range of 51-54 g/kg bw/day. The feed consumption of the scorbutic group was also measured on day 14 and found not to differ from the previous day. This contrasts to the earlier finding that male guinea pigs showed a slight decline in feed intake on day 14 of acute ascorbic acid deficiency (Part VI (2)).

TABLE 24. Effect of acute ascorbic acid deficiency and cholestyramine on body weights, liver weights, and cholesterol and ascorbic acid concentrations in the blood and liver.

The body weights, liver weights and the concentrations of cholesterol in the plasma and liver and of ascorbic acid in the whole blood and liver in all three groups are shown in Table 24. The body weights did not change significantly in any of the groups during the experiment. Although the liver weight in the scorbutic animals was similar to that in the controls the liver weight in the cholestyramine-fed animals was significantly lower ( $P < 0.05$ ). However this largely reflected the difference in body weight, as liver weight expressed on a body weight basis did not differ significantly between the control animals and those fed cholestyramine.

The plasma cholesterol concentration in the control and scorbutic animals did not change significantly during the experiment. This is consistent with the results described in Part VI (2). In the cholestyramine-fed animals there was a significant reduction in plasma cholesterol concentration ( $P < 0.01$ ) as was found in earlier studies (Part V). Also in agreement with earlier studies it was found that the liver cholesterol concentration in the scorbutic and cholestyramine-fed animals was not significantly affected.

The scorbutic group showed significantly lower concentrations of ascorbic acid in the blood ( $P < 0.01$ ) and liver ( $P < 0.001$ ) than the controls. In the cholestyramine-fed animals the blood ascorbic acid concentration did not differ significantly from that in the controls although the liver ascorbic acid concentration was significantly less ( $P < 0.05$ ). The ascorbic acid concentrations found in the control and scorbutic guinea pigs in this study

TABLE 24. Effect of acute ascorbic acid deficiency and cholestyramine feeding on body weights, liver weights and cholesterol and ascorbic acid concentrations in the blood and liver of male guinea pigs<sup>a</sup>

	Diet		
	Plain	Plain	Cholestyramine
Concentration of ascorbic acid in drinking water (% w/v)	0.010	0	0.010
Number of animals	4	4	4
Body weight g			
Initial	762±50.4 <sup>b</sup>	754±28.5	716±20.2
Final	797±46.2	780±32.9	712±14.3
Liver weight g	38.6±1.76	38.1±1.15	31.5±1.58 <sup>*c</sup>
Cholesterol concentration			
Plasma mg/100ml			
Initial	40.7±1.64	37.1±6.10	39.0±5.53
Final	33.2±5.03	26.0±4.75	15.7±2.62 <sup>**d</sup>
Liver mg/g	1.5±0.06	1.6±0.06	1.5±0.06
Ascorbic acid concentration			
Whole blood mg/100ml	0.59±0.051	0.37±0.030 <sup>**c</sup>	0.52±0.033
Liver mg/100g	17.5±1.53	3.5±0.20 <sup>***c</sup>	11.4±1.27 <sup>*c</sup>

<sup>a</sup> Male guinea pigs were fed the plain diet with or without ascorbic acid supplementation or were fed the cholestyramine diet with ascorbic acid supplementation. The group fed the cholestyramine diet and 2 of the animals fed the plain diet with supplementation were killed on day 14 of the experiment. The group fed the plain diet without supplementation and the other 2 animals fed the plain diet with supplementation were killed on day 15.

<sup>b</sup> Values are the mean±S.E. Liver weight and liver cholesterol and ascorbic acid concentrations are expressed on a wet weight basis.

Comparison by Student's t-test:

<sup>c</sup> With corresponding value for animals fed the plain diet and  
<sup>\*</sup> P < 0.05      <sup>\*\*</sup> P < 0.01      <sup>\*\*\*</sup> P < 0.001      supplemented :-

<sup>d</sup> With corresponding initial value :-

<sup>\*\*</sup> P < 0.01



are less than those found in the experiment on the effect of acute scurvy on sterologogenesis (Part VI (2)). It is difficult to account for this difference because the conditions were similar in both studies. However, despite the difference in absolute concentrations of ascorbic acid the scorbutic animals in both studies showed much lower concentrations of ascorbic acid in the blood and liver than the controls.

The results of the assay of cholesterol 7 $\alpha$ -hydroxylase are shown in Table 25. The radioactivity in each band was expressed as a percentage of the total radioactivity recovered from all six bands. The recovery of activity from each chromatographic separation averaged  $84.8 \pm 1.04\%$  (range 75.9-99.0) of the total radioactivity added to the incubation mixture. In the control and scorbutic animals the percentage of activity recovered in the 7 $\alpha$ -hydroxycholesterol band from those incubations with fresh supernatant was similar to that from those incubations with boiled supernatant. In the cholestyramine-fed animals the percentage of radioactivity recovered in the 7 $\alpha$ -hydroxycholesterol band from the incubations with fresh supernatant was about twice that from the incubations with boiled supernatant. However as the level of conversion was extremely low it is doubtful that this higher percentage recovery was indicative of enzyme activity. In all incubations approximately 97% of the radioactivity recovered from the plate was present in the cholesterol band. To obtain a measure of the autoxidation which resulted during the extraction and separation procedures, two zero-time, non-incubated

TABLE 25. Effect of acute ascorbic acid deficiency and cholestyramine feeding on cholesterol 7 $\alpha$ -hydroxylase activity in male guinea pigs<sup>a</sup>

Concentration of ascorbic acid in drinking water (% w/v)	Diet					
	Plain		Plain		Cholestyramine	
	0.010		0		0.010	
Number of animals	4		4		4	
Region of chromatographic plate corresponding to <sup>c</sup> :	Enzyme preparation <sup>b</sup>					
	Fresh		Boiled		Fresh	
Origin	0.24 $\pm$ 0.024 <sup>d</sup>	0.27 $\pm$ 0.038	0.24 $\pm$ 0.019	0.27 $\pm$ 0.025	0.28 $\pm$ 0.017	0.27 $\pm$ 0.017
Polar metabolite <sup>e</sup>	0.19 $\pm$ 0.019	0.10 $\pm$ 0.007	0.20 $\pm$ 0.019	0.11 $\pm$ 0.007	0.18 $\pm$ 0.017	0.11 $\pm$ 0.020
7 $\alpha$ -hydroxycholesterol	0.29 $\pm$ 0.031	0.31 $\pm$ 0.026	0.27 $\pm$ 0.051	0.27 $\pm$ 0.051	0.64 $\pm$ 0.176	0.30 $\pm$ 0.030
7 $\beta$ -hydroxycholesterol	0.77 $\pm$ 0.041	0.62 $\pm$ 0.040	0.74 $\pm$ 0.099	0.68 $\pm$ 0.015	0.47 $\pm$ 0.056	0.58 $\pm$ 0.088
7-ketocholesterol	1.09 $\pm$ 0.161	1.41 $\pm$ 0.184	1.11 $\pm$ 0.142	1.55 $\pm$ 0.239	1.70 $\pm$ 0.115	1.75 $\pm$ 0.303
Cholesterol	97.42 $\pm$ 0.122	97.29 $\pm$ 0.218	97.44 $\pm$ 0.137	97.12 $\pm$ 0.268	96.73 $\pm$ 0.186	96.99 $\pm$ 0.290

<sup>a</sup>See footnote a of Table 24.

<sup>b</sup>The source of cholesterol 7 $\alpha$ -hydroxylase was the 18,000xg supernatant. For each animal three incubations were carried out. Two contained fresh supernatant and the third boiled supernatant.

<sup>c</sup>The thin-layer chromatographic separation is described in Figure 2.

<sup>d</sup>The radioactivity in each respective band was expressed as a percentage of the total radioactivity recovered from all six bands. The mean recovery of the total radioactivity originally added to each incubation was 84.8 $\pm$ 1.04% (range 75.9-99.0). Values for the fresh enzyme preparation are the mean $\pm$ S.E. calculated using the average values for the duplicate incubations. Values for the boiled enzyme preparation are the mean $\pm$ S.E. calculated using values from single incubations.

<sup>e</sup>The polar metabolite is derived from 7-ketocholesterol and is possibly a 12-hydroxylated derivative (Van Cantfort *et al.*, 1975).

blanks were prepared. In these, fresh supernatant from control animals was used and the reaction was stopped immediately following the addition of the labelled substrate. For these blanks the mean recovery of the radioactivity added to the reaction mixture was 73% and the amount of activity in the 7 $\alpha$ -hydroxycholesterol band averaged 0.19% of the total radioactivity recovered from all six bands.

(b) Discussion.

There is substantial evidence to show that cholesterol 7 $\alpha$ -hydroxylase under normal conditions is the rate-limiting enzyme in the major pathway for the synthesis of bile acids from cholesterol (Danielsson et al., 1967; Shefer et al., 1970). The enzyme is located in the microsomal fraction (Shefer et al., 1968; Nicolau et al., 1974) and as shown by the studies of Mitton et al. (1971) and Aringer and Eneroth (1973) cholesterol 7 $\alpha$ -hydroxylase could be readily measured in the 18,000xg supernatant fraction of rat liver. Mitton et al. (1971) found higher enzyme activity in this fraction than in the microsomal fraction. Thus in the present study an attempt was made to measure cholesterol 7 $\alpha$ -hydroxylase in guinea pig liver using a procedure similar to that of Mitton et al. (1971). The main modification to their method was that the substrate, 4-<sup>14</sup>C-cholesterol, was suspended in Tween 80 and not dissolved in acetone.

In rats the activity of cholesterol 7 $\alpha$ -hydroxylase shows a diurnal variation which peaks at about the middle of the dark period (Mitropoulos et al., 1972). All guinea pigs were therefore killed at approximately this time.



Studies by Kritchevsky et al. (1973) have demonstrated that in normal animals the specific activity of cholesterol 7 $\alpha$ -hydroxylase in rat liver microsomes is approximately 4 times that in guinea pig liver microsomes. Thus as Mitton et al. (1971) found that the 18,000xg supernatant of rat liver converted up to 10% of labelled cholesterol to 7 $\alpha$ -hydroxycholesterol under optimal conditions, it was expected that the supernatant of livers from the control guinea pigs would have shown up to 2.5% conversion. A much higher conversion was expected with the supernatants from the livers of guinea pigs fed cholestyramine as such treatment in rats enhances the activity of cholesterol 7 $\alpha$ -hydroxylase several fold (Shefer et al., 1968; Boyd et al., 1973; Mitropoulos et al., 1973). However it is apparent that no enzyme activity was obtained in any of the three groups of animals. Only about 0.3% of the 4-<sup>14</sup>C-cholesterol was converted to 7 $\alpha$ -hydroxycholesterol by the fresh supernatants from the control and scorbutic animals. As a similar percentage conversion occurred with the boiled supernatants it appears that all the labelled 7 $\alpha$ -hydroxycholesterol was formed by autoxidation. In the assay of cholesterol 7 $\alpha$ -hydroxylase in human hepatic microsomes Nicolau et al. (1974) found that in boiled enzyme blanks the conversion of labelled cholesterol to 7 $\alpha$ -hydroxycholesterol was 20-30% of that obtained with active enzyme preparations. In the present experiments much of the autoxidation must have occurred during the extraction and separation procedures because in the zero-time non-incubated blanks 0.19% of the total radioactivity recovered from each chromatographic mixture.

separation appeared in 7 $\alpha$ -hydroxycholesterol. As the substrate, 4-<sup>14</sup>C-cholesterol, was purified immediately before use, the amount of labelled 7 $\alpha$ -hydroxycholesterol initially added to the reaction mixtures was probably negligible.

It is unlikely that any loss of enzyme activity occurred during the preparation of the 18,000xg supernatant because the procedure used was similar to that described by Mitton et al. (1971). The only modification to this procedure was that the initial centrifugation of the homogenate at 600xg for 10 min was not carried out.

In the studies by Mitton et al. (1971) and others (Danielsson et al., 1967; Aringer and Eneroth, 1973) acetone was used to introduce the substrate into the incubation mixture. In preliminary studies no enzyme activity could be detected in supernatants from guinea pig liver when the 4-<sup>14</sup>C-cholesterol was added to the incubation mixture in acetone. It was thus decided to use Tween 80 as the medium. The suspension of labelled cholesterol in either Tween 80 or Tween 20 has been widely used in the assay of cholesterol 7 $\alpha$ -hydroxylase in liver preparations from humans (Nicolau et al., 1974), guinea pigs (Kritchevsky et al., 1973) and rats (Mitropoulos et al., 1973; Van Cantfort et al., 1975). In the present experiments the final concentration of Tween 80 in the incubation mixture (approximately 0.5 mg/ml) was in the optimal range described by Van Cantfort et al. (1975). Therefore it is unlikely that the failure to obtain enzyme activity in the present studies resulted because of the medium used to introduce the substrate into the reaction mixture.

In the present experiments the concentrations of exogenous and endogenous cholesterol in the incubation mixture were approximately 2  $\mu\text{M}$  and 260  $\mu\text{M}$  respectively. This concentration of exogenous cholesterol is greatly below that which has been shown to be required to give maximal rates of cholesterol 7 $\alpha$ -hydroxylase in the hepatic microsomal fraction from other species. In human hepatic microsomes the optimum concentration of exogenous cholesterol was shown to be approximately 100  $\mu\text{M}$  (Nicolau et al., 1974). In rat liver microsomes maximal rates of 7 $\alpha$ -hydroxylase activity occurred at an exogenous cholesterol concentration of approximately 300  $\mu\text{M}$ , this being about 4 times the concentration of endogenous cholesterol (Van Cantfort et al., 1975). Thus it may appear that the failure to obtain enzyme activity in the present experiments resulted because the concentration of exogenous cholesterol was too low relative to the concentration of endogenous cholesterol. However in the studies of Mitton et al. (1971) and Aringer and Eneroth (1973) high levels of conversion of labelled cholesterol to 7 $\alpha$ -hydroxycholesterol were obtained with concentrations of exogenous cholesterol which were approximately 0.6  $\mu\text{M}$  and 2.9  $\mu\text{M}$  respectively. These concentrations are similar to that used in the present study. It would however be useful to examine the conversion rates of labelled cholesterol to 7 $\alpha$ -hydroxycholesterol by guinea pig liver preparations at an exogenous cholesterol concentration about 100 times that used in the present study.

Although the present experiments have failed to establish whether dietary ascorbic acid deficiency results



in decreased cholesterol 7 $\alpha$ -hydroxylase activity, the newly published findings of Björkhem and Kallner (1976) have clearly demonstrated such an effect. Using a mass fragmentographic method to measure the 7 $\alpha$ -hydroxylation of endogenous cholesterol they showed that in hepatic microsomes from guinea pigs with chronic scurvy, the activity of cholesterol 7 $\alpha$ -hydroxylase was 15 times less than in microsomes from guinea pigs which had received adequate ascorbic acid supplementation. It is likely however, that in all animals the activity of cholesterol 7 $\alpha$ -hydroxylase was overestimated because it appears that no boiled enzyme blanks were included. Björkhem and Kallner also showed that the addition of ascorbic acid at a final concentration of 0.4 mM had no effect on cholesterol 7 $\alpha$ -hydroxylase activity in microsomes from either scorbutic or normal guinea pigs. Kritchevsky et al. (1973) had earlier reported that the incubation of hepatic microsomes from normal guinea pigs and rats with ascorbic acid at concentrations up to approximately 0.4 mM did not significantly affect cholesterol 7 $\alpha$ -hydroxylase activity. In contrast Van Cantfort et al. (1975) reported that incubation of rat liver microsomes in the presence of 10 mM ascorbic acid enhanced the production of 7 $\alpha$ -hydroxycholesterol. However this appears to have resulted from an effect of ascorbic acid in limiting the formation of autoxidation products such as 7 $\beta$ -hydroxycholesterol and 7-ketocholesterol rather than by directly stimulating cholesterol 7 $\alpha$ -hydroxylase activity.

Björkhem and Kallner (1976) also found that in the presence of excess NADPH-cytochrome P-450 reductase

and a phospholipid, partially purified cytochrome P-450 from the microsomal fraction of liver of an ascorbic acid-deficient guinea pig had a much lower capacity to 7 $\alpha$ -hydroxylate 4-<sup>14</sup>C-cholesterol than a corresponding system containing cytochrome P-450 from liver of a normal guinea pig. They have therefore suggested that ascorbic acid affects the synthesis or breakdown of the 7 $\alpha$ -hydroxylating system, in particular the cytochrome P-450 component. This is entirely consistent with the finding of Ginter and Nemec (1972) that the time course of the enhancement of the oxidation of 26-<sup>14</sup>C-cholesterol to <sup>14</sup>CO<sub>2</sub> produced by injecting ascorbic acid into chronically scorbutic guinea pigs closely parallels the increase in the concentration of cytochrome P-450 observed under these conditions by Leber et al. (1970).

Although it has now been established that ascorbic acid is involved in the regulation of cholesterol 7 $\alpha$ -hydroxylase activity, it is possible that other sites in the pathway of the conversion of cholesterol to bile acids, such as side chain oxidation in the mitochondria, are regulated by ascorbic acid. Guchhait et al. (1963) showed that the formation of bile acids from 4-<sup>14</sup>C-cholesterol by guinea pig mitochondria was enhanced by the addition of ascorbic acid in vitro. The effect was more pronounced with mitochondria from acutely scorbutic animals than with those from pair-fed controls. However Kritchevsky et al. (1973) were not able to demonstrate any stimulatory effect of ascorbic acid on the oxidation of 26-<sup>14</sup>C-cholesterol by mitochondria from normal guinea pigs or rats; probably because the mitochondrial preparations already contained

the optimum concentration of ascorbic acid. Neither of these studies take into account that the  $7\alpha$ -hydroxylation of cholesterol which is the initial step in the primary route for the conversion of cholesterol to bile acids, occurs primarily in the microsomes. Shefer et al. (1968) have shown in studies with rats, that microsomes, on a protein-weight basis, have more than 11 times the  $7\alpha$ -hydroxylating activity than do mitochondria. Thus the role of mitochondria should be tested not with cholesterol but with steroids known to serve as mitochondrial substrates such as  $5\beta$ -cholestane- $3\alpha$ ,  $7\alpha$ -diol (Boyd and Percy-Robb, 1971).

An alternative pathway of bile acid synthesis has been suggested by Mitropoulos and Myant (1967a,b) in which cholesterol is first converted to 26-hydroxycholesterol. It is possible that the effect of ascorbic acid on bile acid synthesis reported by Guchhait et al. (1963) was on this pathway and not on that in which  $7\alpha$ -hydroxycholesterol is first formed. Although Björkhem and Kallner (1976) found that 25- and 26-hydroxylation of  $5\beta$ -cholestane- $3\alpha$ ,  $7\alpha$ -diol did not differ in the microsomes from scorbutic and normal guinea pigs, it would have been more appropriate if the studies had been carried out using the mitochondria from these animals.

Although the present study was unable to establish the extent to which cholestyramine feeding stimulated bile acid synthesis in the guinea pig, it did demonstrate that such treatment resulted in a significant decrease in the ascorbic acid concentration in the liver. If cholestyramine feeding stimulated cholesterol  $7\alpha$ -hydroxylase several fold



in guinea pigs as it does in rats (Shefer et al., 1968; Boyd et al., 1973; Mitropoulos et al., 1973), then the lower hepatic ascorbic acid concentrations could be explained partly by an increased demand for ascorbic acid in the enhanced production of bile acids. Ascorbic acid appears to also be involved in cholesterol excretion (Verlangieri and Mumma, 1973). Thus as liver sterol production is greatly enhanced in guinea pigs fed with cholestyramine (Part V), there may have been an increased demand for ascorbic acid in the excretion of this sterol. In these studies the guinea pigs consumed approximately 1.5 g cholestyramine/kg bw/day. Danhof (1966) showed that in normocholesterolaemic humans maximum reduction in plasma cholesterol concentration was achieved by consuming 12 g cholestyramine/day. This is equivalent to about 0.2 g/kg bw/day which is much less than the dose given to the guinea pigs. However as the use of cholestyramine for the treatment of hypercholesterolaemia in humans requires consumption of the resin over long periods, it is possible that such patients would need to supplement their diet with ascorbic acid, particularly if their intake at the beginning of treatment was only equivalent to, or less than, the normal daily requirement. on serum cholesterol concentration. Similar studies in animals on normal diets appear not to have been made although in normocholesterolaemic humans the ingestion of ascorbic acid in amounts greatly exceeding the daily requirement over long periods is without effect on plasma cholesterol concentration (Anderson et al., 1958; Sokoloff et al., 1966; Crawford et al., 1975).

PART VI (4). Effects of high levels of ascorbic acid supplementation on cholesterol metabolism in guinea pigs.

Most studies on the effects of a high intake of vitamin C on plasma and tissue cholesterol concentrations have been carried out using animals fed atherogenic diets. In rats (Sokoloff et al., 1967) and rabbits (Sokoloff et al., 1967; Ginter et al., 1970a) supplementation with high levels of ascorbic acid markedly reduces dietary cholesterol-induced hypercholesterolaemia. However, as cholesterol feeding in both these species stimulates ascorbic acid biosynthesis (Ginter, 1970), the effects of administering additional ascorbic acid on plasma cholesterol concentrations cannot be extrapolated to those species which are unable to synthesise ascorbic acid. Ginter and his colleagues have examined the effect of different levels of ascorbic acid supplementation on serum and tissue cholesterol concentrations in guinea pigs fed a cholesterol diet over several months (Ginter et al., 1969a,b,d). Although high levels of ascorbic acid supplementation significantly reduced the accumulation of cholesterol in several tissues including the liver and adrenals, there was no effect on serum cholesterol concentration. Similar studies in animals on normal diets appear not to have been made although in normocholesterolaemic humans the ingestion of ascorbic acid in amounts greatly exceeding the daily requirement over long periods is without effect on plasma cholesterol concentration (Anderson et al., 1958; Sokoloff et al., 1966; Crawford et al., 1975).

The addition of ascorbic acid to liver preparations from normal baboons has no effect on cholesterol synthesis (Weight et al., 1974). Similarly the activity of cholesterol 7 $\alpha$ -hydroxylase is not significantly affected when ascorbic acid is added to microsomal preparations from the livers of normal guinea pigs (Kritchevsky et al., 1973). The lack of such effects in vitro however does not exclude the possibility that cholesterol and bile acid synthesis are influenced by the prolonged high intake of ascorbic acid.

Thus the present series of experiments examined the effects of high levels of ascorbic acid supplementation on cholesterol metabolism in male and female guinea pigs.

(a) Results.

In the first experiment the effect of supplementing female guinea pigs with 10 times their normal daily requirement of ascorbic acid for 5 months on plasma and tissue cholesterol and ascorbic acid concentrations and hepatic and intestinal sterologenesi, was examined. Cholesterol 7 $\alpha$ -hydroxylase activity was not measured because of difficulties with the assay.

The experiments described in Part VI (1) demonstrated that the plasma cholesterol concentration in guinea pigs markedly increased when they were transferred from the breeding room to the light-controlled room. As shown in Figure 20, supplementation with ascorbic acid at 10 times the daily requirement did not significantly affect this increase. In both groups the plasma cholesterol concentration at 4 weeks was significantly higher ( $P < 0.01$  for control group and



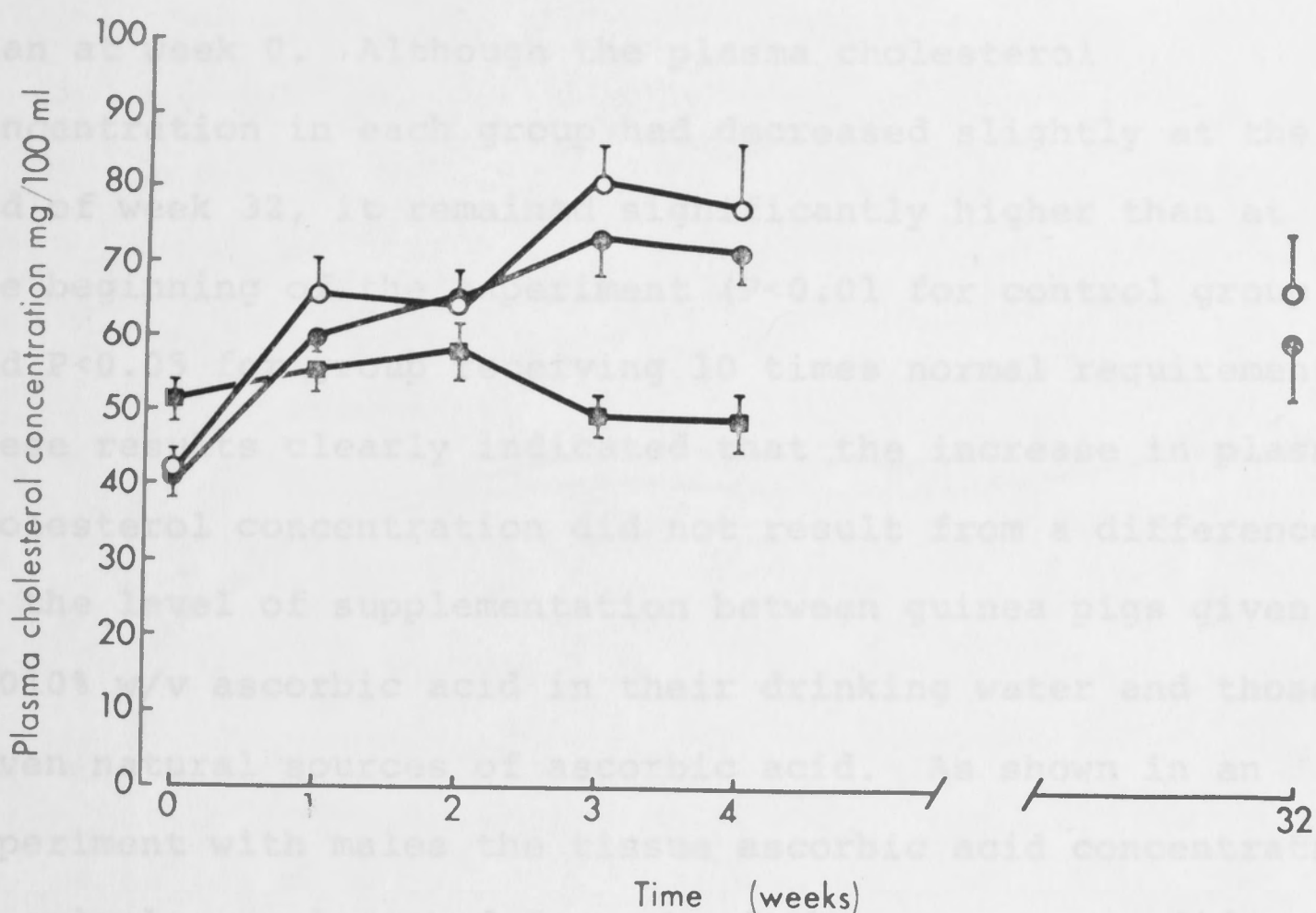


Figure 20. Effect of the level of ascorbic acid supplementation on the change in plasma cholesterol concentration observed in guinea pigs when transferred to the light-controlled room. Two studies on female guinea pigs were carried out. In the first, the plasma cholesterol concentration of two groups of 5 animals was determined while they were housed in the room where they had been bred. They were then transferred to the light-controlled room where one group received water containing 0.010% w/v ascorbic acid (○—○) and the other, water containing 0.100% w/v ascorbic acid (●—●). Plasma cholesterol concentration was again determined for the following 4 weeks and at the end of the study. In the second study, carried out about 2 months after the completion of the first, the plasma cholesterol concentration was determined in 10 animals over a 4-week period while they remained in the room where they had been bred (■—■). These animals received natural sources of ascorbic acid supplementation. Values are the mean  $\pm$  S.E.

$P < 0.001$  for group receiving 10 times normal requirement) than at week 0. Although the plasma cholesterol concentration in each group had decreased slightly at the end of week 32, it remained significantly higher than at the beginning of the experiment ( $P < 0.01$  for control group and  $P < 0.05$  for group receiving 10 times normal requirement). These results clearly indicated that the increase in plasma cholesterol concentration did not result from a difference in the level of supplementation between guinea pigs given 0.010% w/v ascorbic acid in their drinking water and those given natural sources of ascorbic acid. As shown in an experiment with males the tissue ascorbic acid concentrations of animals supplemented in each of these two ways, did not differ significantly (Part VI (2)).

Unlike the guinea pigs transferred to the light-controlled room, those remaining in the breeding room did not show a significant change in plasma cholesterol concentration when bled at weekly intervals for 4 weeks (Figure 20). This suggested that the increase in plasma cholesterol concentration in guinea pigs in the light-controlled room was not the result of stress associated with bleeding at weekly intervals. Thus the increase probably resulted from a combination of a disturbance in the social order of the animals, maintenance under controlled lighting and the elimination from their diet of supplementary pumpkin and fodder. As plasma cholesterol concentrations had stabilised by approximately 3 weeks, all studies except that described in Part VI (1) were carried out after an adjustment period of at least 18 days.

As shown in Figure 21, the body weight gain of

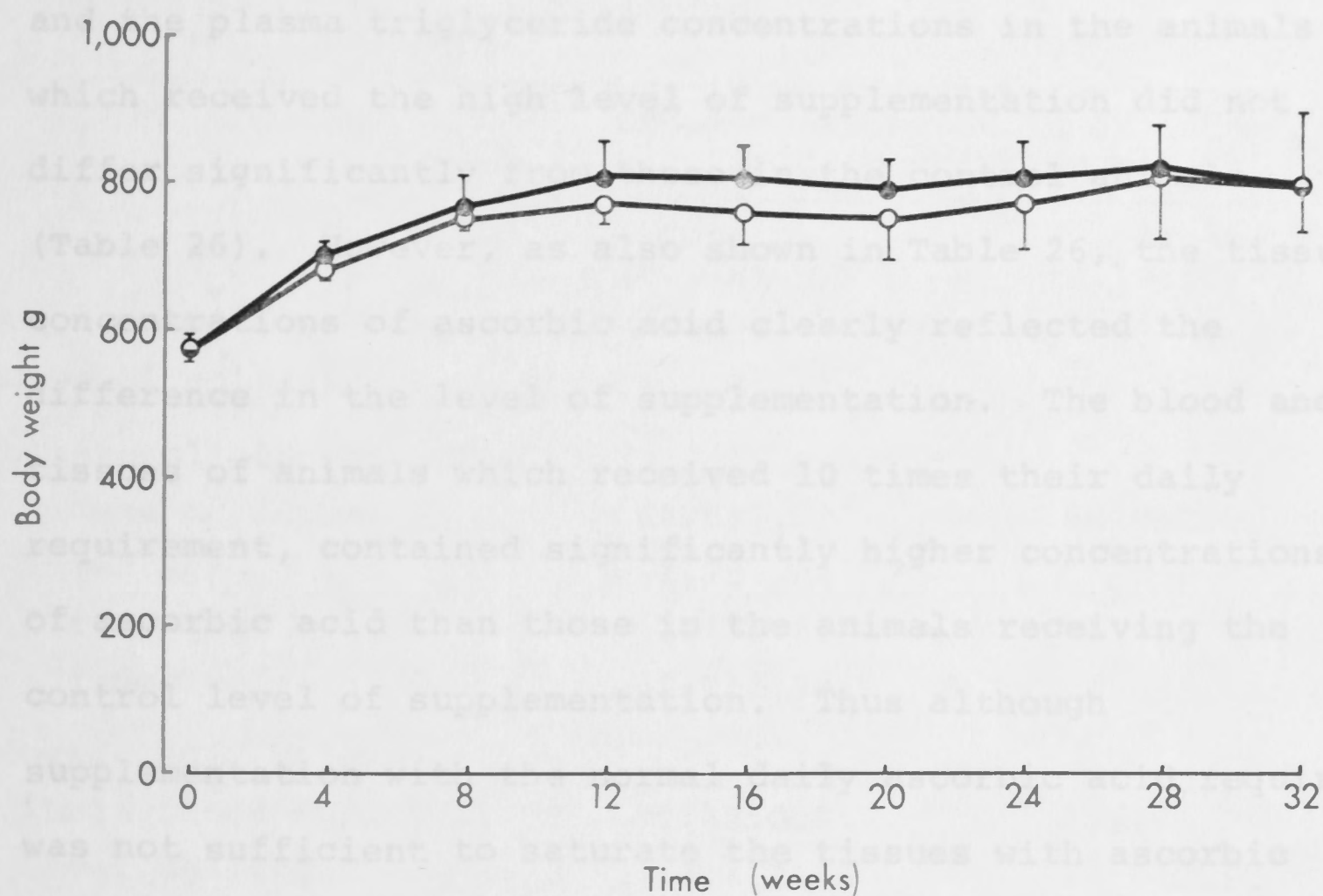


Figure 21. Body weights of female guinea pigs receiving different levels of ascorbic acid supplementation. Two groups of 5 animals fed the plain diet were given water containing either 0.010% w/v (○—○) or 0.100% w/v (●—●) ascorbic acid. Their body weights were recorded weekly for 32 weeks. Only the values at 4-weekly intervals are shown. Values are the mean±S.E.

In the second experiment the effect of different levels of ascorbic acid supplementation on the plasma cholesterol concentrations in female guinea pigs fed either the cholesterol or plain diet over an 11-week period was studied (Figure 22). During the first 22 days of the experiment when both groups received the optimal level of ascorbic acid supplementation, the plasma cholesterol



animals receiving 10 times their normal ascorbic acid requirement did not differ from that of animals receiving the control level of supplementation.

The plasma and tissue cholesterol concentrations and the plasma triglyceride concentrations in the animals which received the high level of supplementation did not differ significantly from those in the control animals (Table 26). However, as also shown in Table 26, the tissue concentrations of ascorbic acid clearly reflected the difference in the level of supplementation. The blood and tissues of animals which received 10 times their daily requirement, contained significantly higher concentrations of ascorbic acid than those in the animals receiving the control level of supplementation. Thus although supplementation with the normal daily ascorbic acid requirement was not sufficient to saturate the tissues with ascorbic acid, it was sufficient to maintain normal concentrations of cholesterol and triglyceride.

As shown in Table 27 supplementation with 10 times the normal ascorbic acid requirement did not significantly affect either hepatic or intestinal sterologenesis. This was consistent with the lack of effect on tissue cholesterol concentrations.

In the second experiment the effect of different levels of ascorbic acid supplementation on the plasma cholesterol concentrations in female guinea pigs fed either the cholesterol or plain diet over an 11-week period was studied (Figure 22). During the first 22 days of the experiment when both groups received the optimal level of ascorbic acid supplementation, the plasma cholesterol

TABLE 26. Effect of prolonged high intake of ascorbic acid on cholesterol and ascorbic acid concentrations in the plasma and tissues and on triglyceride concentration in the plasma of female guinea pigs<sup>a</sup>.

	Group (concentration of ascorbic acid in drinking water)	
	Control (0.010% w/v)	High (0.100% w/v)
Number of animals	4	5
Cholesterol concentration		
Plasma mg/100 ml	68.0±7.87 <sup>b</sup>	60.8±7.36
Liver mg/g	1.7±0.08	1.7±0.08
Ileum mg/g	1.7±0.07	1.8±0.09
Adrenal mg/g	58.2±3.96	64.6±5.38
Ascorbic acid concentration		
Whole blood mg/100 ml	0.42±0.058	1.97±0.288 <sup>**c</sup>
Liver mg/100g	14.2±2.24	53.4±2.83 <sup>***</sup>
Ileum mg/100g	12.8±1.53	35.8±2.20 <sup>***</sup>
Adrenal mg/100g	62.8±9.82	122.1±7.80 <sup>**</sup>
Triglyceride concentration		
Plasma mg/100ml	114.0±6.26	130.0±8.17

<sup>a</sup>Two groups of female guinea pigs fed the plain diet, received different levels of ascorbic acid supplementation as described in Figure 20. All animals were killed at the end of the 32nd week of the experiment.

<sup>b</sup>Values are the mean±S.E. Tissue cholesterol and ascorbic acid concentrations are expressed on a wet weight basis.

<sup>c</sup>Comparison by Student's t-test with corresponding value for control group:

<sup>\*\*</sup>P<0.01

<sup>\*\*\*</sup>P<0.001

TABLE 27. Effect of prolonged high intake of ascorbic acid on sterol synthesis in the liver and ileum of female guinea pigs<sup>a</sup>.

Group (Concentration of ascorbic acid in drinking water)	Number of animals	Sterol synthesis from 1- <sup>14</sup> C-acetate mμmoles incorporated/g tissue/h <sup>b</sup>		Sterol synthesis from 2- <sup>14</sup> C-mevalonate mμmoles incorporated/g tissue/h	
		Liver	Ileum	Liver	Ileum
Control (0.010% w/v)	4	7.4±2.03 <sup>c</sup>	73.2±5.70	36.5±6.00	94.7±11.69
High (0.100% w/v)	5	10.4±2.89	74.1±8.42	38.7±7.03	87.0±7.45

<sup>a</sup> See footnote a of Table 26.

<sup>b</sup> Slices of liver and ileum were incubated in triplicate in Krebs-Ringer phosphate buffer containing either 1-<sup>14</sup>C-acetate or 2-<sup>14</sup>C-mevalonate.

<sup>c</sup> Values are the mean±S.E.

Differences between groups, as compared by Student's t-test, are not statistically significant.



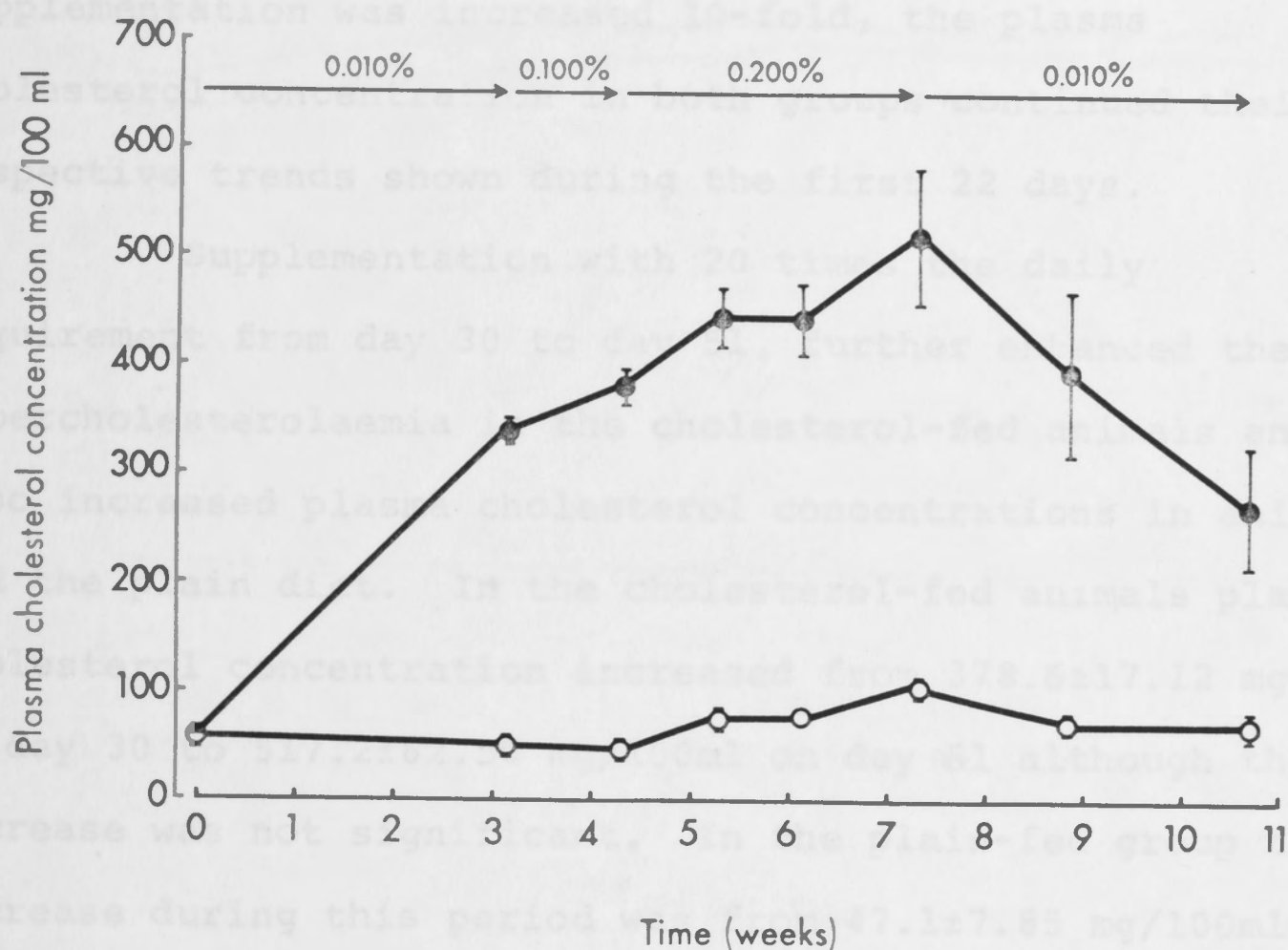


Figure 22. Plasma cholesterol concentration in female guinea pigs fed either the plain or cholesterol diet during a period in which the level of ascorbic acid supplementation was varied. One group of animals received the plain diet (○—○) while another group was given a diet containing 0.24% w/w cholesterol (●—●). The concentration of ascorbic acid in the drinking water of both groups was varied throughout the experiment as indicated. Concentrations are expressed as % w/v. There were 4 animals in the group receiving the plain diet and 5 in the group receiving the cholesterol diet. Values are the mean  $\pm$  S.E.

concentration of the cholesterol-fed group increased greatly while that of the group fed the plain diet remained constant. During the ensuing 8 days when the level of supplementation was increased 10-fold, the plasma cholesterol concentration in both groups continued their respective trends shown during the first 22 days.

Supplementation with 20 times the daily requirement from day 30 to day 51, further enhanced the hypercholesterolaemia in the cholesterol-fed animals and also increased plasma cholesterol concentrations in animals fed the plain diet. In the cholesterol-fed animals plasma cholesterol concentration increased from  $378.6 \pm 17.12$  mg/100ml on day 30 to  $517.2 \pm 62.50$  mg/100ml on day 51 although this increase was not significant. In the plain-fed group the increase during this period was from  $47.1 \pm 7.85$  mg/100ml to  $105.9 \pm 11.05$  mg/100ml ( $P < 0.01$ ). After day 51 when the ascorbic acid supplementation was returned to normal, the plasma cholesterol concentration in both groups decreased. However only in the cholesterol-fed group was the decrease from day 51 to day 75 significant ( $P < 0.05$ ).

Throughout the experiment the body weight gains of both groups did not differ significantly (Figure 23). However as shown in Table 28 cholesterol feeding resulted in a significantly higher liver weight ( $P < 0.01$ ) but adrenal weight was not significantly different. In addition to increasing the plasma cholesterol concentration, cholesterol feeding significantly increased the cholesterol concentration in the liver ( $P < 0.05$ ) and adrenals ( $P < 0.05$ ) (Table 28). The liver cholesterol concentration in the cholesterol-fed animals was 9 times that in the animals fed the plain diet.

TABLE 23. Organ weights, and concentrations and total contents of cholesterol and ascorbic acid in the plasma and

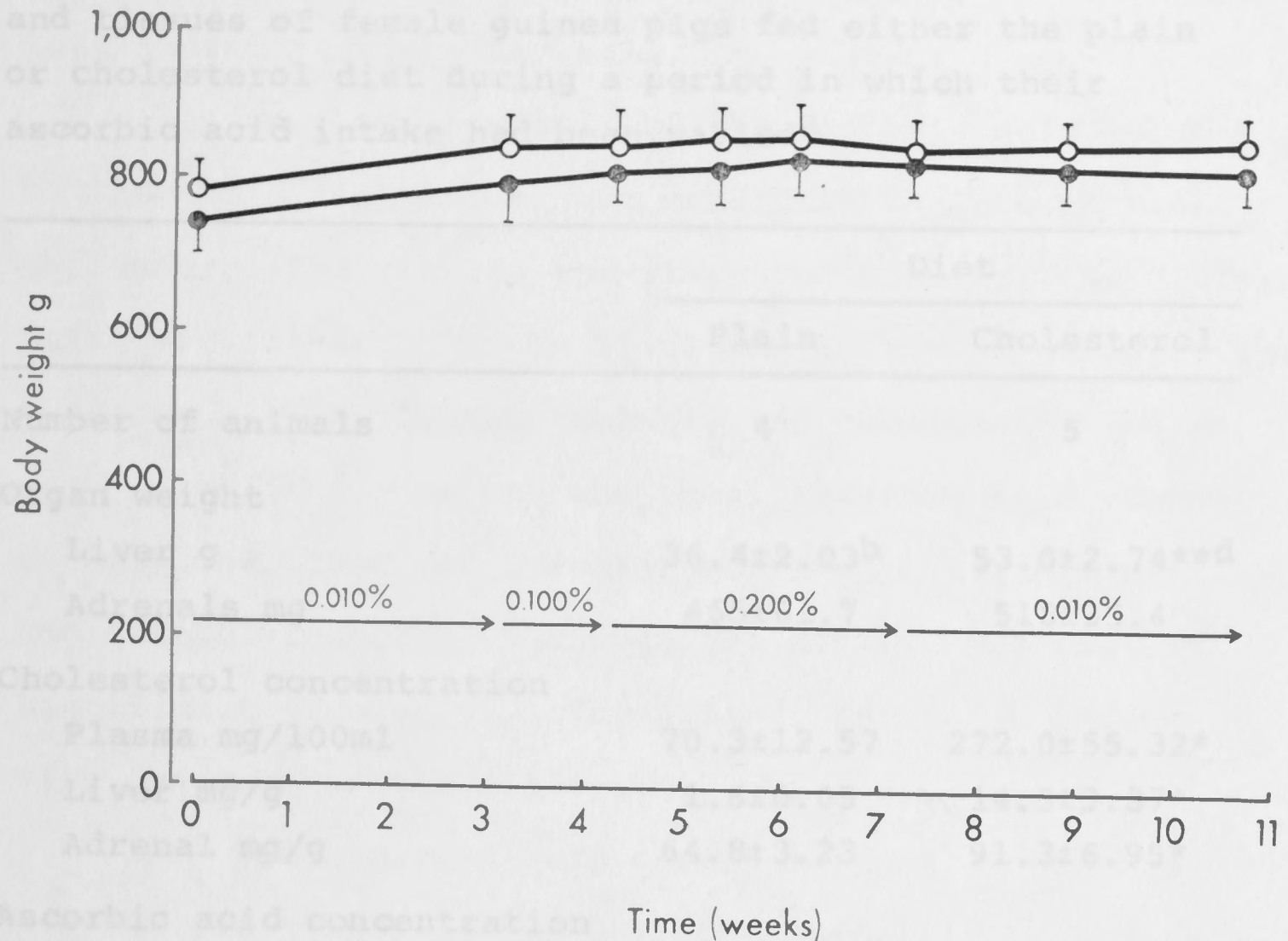


Figure 23. Body weights of female guinea pigs fed either the plain or cholesterol diet during a period in which the level of ascorbic acid supplementation was varied. One group of animals received the plain diet (○—○) while another group was given a diet containing 0.24% w/w cholesterol (●—●). The concentration of ascorbic acid in the drinking water of both groups was varied throughout the experiment as indicated. Concentrations are expressed as % w/v. There were 4 animals in the group receiving the plain diet and 5 in the group receiving the cholesterol diet. Values are the mean ± S.E.

<sup>a</sup> The level of ascorbic acid supplementation was varied as described in Figure 22.

<sup>b</sup> Values are the mean ± S.E. and are expressed on a wet weight basis.

<sup>c</sup> Determinations from 4 animals only.

<sup>d</sup> Comparison by Student's t-test with corresponding value for animals receiving plain diet.

\* $p < 0.05$  \*\* $p < 0.01$



TABLE 28. Organ weights, and concentrations and total contents of cholesterol and ascorbic acid in the plasma and tissues of female guinea pigs fed either the plain or cholesterol diet during a period in which their ascorbic acid intake had been varied<sup>a</sup>.

	Diet	
	Plain	Cholesterol
Number of animals	4	5
Organ weight		
Liver g	36.4±2.03 <sup>b</sup>	53.0±2.74**d
Adrenals mg	465±61.7	518±35.4
Cholesterol concentration		
Plasma mg/100ml	70.3±12.57	272.0±55.32*
Liver mg/g	1.6±0.05	14.5±3.37*
Adrenal mg/g	64.8±3.23	91.3±6.95*
Ascorbic acid concentration		
Whole blood mg/100ml	1.02±0.590	0.51±0.074 <sup>c</sup>
Liver mg/100g	34.6±7.75	16.3±1.86*
Adrenal mg/100g	96.1±12.74	51.3±7.03*
Total cholesterol content		
Liver mg	59.2±3.13	769±201.0*
Adrenals mg	29.9±4.11	47.5±5.45*
Total ascorbic acid content		
Liver mg	12.2±2.33	8.7±1.24
Adrenals mg	0.45±0.086	0.27±0.049

<sup>a</sup> The level of ascorbic acid supplementation was varied as described in Figure 22.

<sup>b</sup> Values are the mean±S.E. and are expressed on a wet weight basis.

<sup>c</sup> Determinations from 4 animals only.

<sup>d</sup> Comparison by Student's t-test with corresponding value for animals receiving plain diet:

\*P<0.05

\*\*P<0.01

The total cholesterol content of both the liver and adrenals was significantly higher ( $P < 0.05$ ) in the cholesterol-fed than in the plain-fed animals.

As shown in Table 28 the ascorbic acid concentration in the blood and tissues was considerably less in the cholesterol-fed than in the plain-fed animals, the difference being significant for the liver and adrenal ( $P < 0.05$ ). Even when the higher organ weights in the cholesterol-fed animals were taken into account, the total ascorbic acid content of both the liver and adrenal was much less in the cholesterol-fed group although the differences are not significant (Table 28). These findings are in agreement with those of Ginter (1970).

The ascorbic acid concentrations of the group fed the plain diet (Table 28) are noticeably higher than in the first experiment in which females were also used (Table 26). However this difference almost certainly was only a direct result of the high levels of supplementation given in the present experiment. In the earlier experiment the level of supplementation was maintained at 0.010% w/v over the 32-week period.

Although the first experiment had shown that ascorbic acid supplementation at 10 times the normal requirement had no effect on plasma cholesterol concentration in normocholesterolaemic female guinea pigs, the results of the second experiment showed that when the supplementation was increased to 20 times the daily requirement, plasma cholesterol concentration was increased. Thus a third experiment using male guinea pigs fed the plain diet was carried out to compare the effects of supplementation at

10 times the normal ascorbic acid requirement with supplementation at 20 and later 40 times normal on plasma cholesterol concentration. The body weight gains of both groups were not significantly different and resembled those of males receiving normal levels of vitamin C supplementation in other experiments. As shown in Figure 24, the plasma cholesterol concentration of the group receiving 20 and later 40 times the normal ascorbic acid requirement did not differ significantly from that in the group given 10 times the normal requirement throughout the same period. Furthermore the plasma cholesterol concentration in both groups remained within the range generally observed in males maintained in the light-controlled room.

The ascorbic acid concentrations in the blood and liver of the group which received 0.400% w/v ascorbic acid in their drinking water were not significantly different from those in the group which had been given 0.100% w/v ascorbic acid suggesting that this level of supplementation was sufficient to saturate the blood and tissues with ascorbic acid (Table 29). It is unlikely that any change in plasma cholesterol concentration would have occurred if the high level of supplementation had been continued; in a very recent study by Nambisan and Kurup (1975) it was shown that the plasma cholesterol concentration of normocholesterolaemic male guinea pigs was unaffected after 4 months of a high level of ascorbic acid supplementation. Ginter *et al.* (1969a,b,d) had earlier shown that such treatment also had no effect on plasma cholesterol concentration in male guinea pigs with dietary cholesterol-induced hypercholesterolaemia.



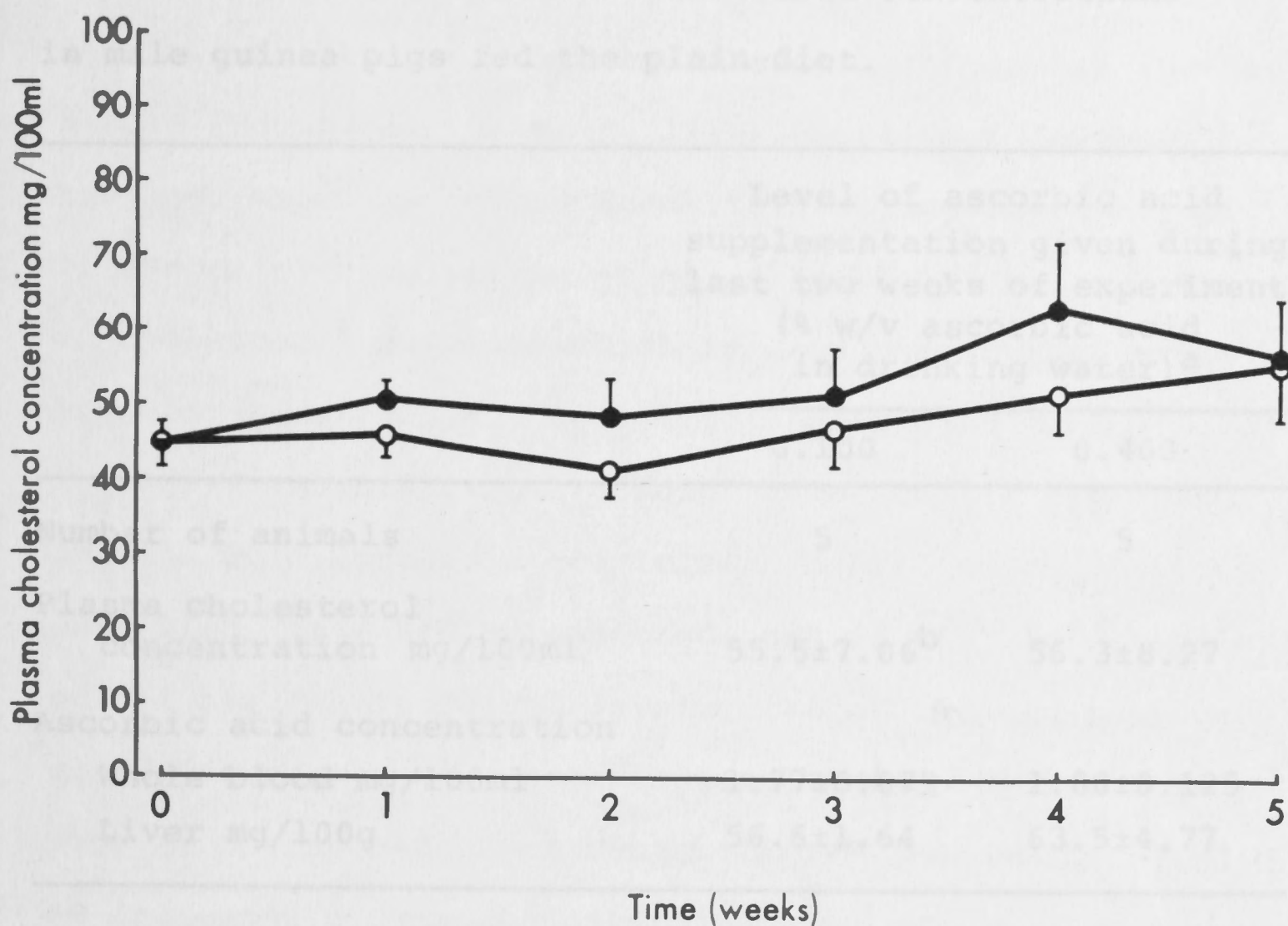


Figure 24. Effect of high levels of ascorbic acid supplementation on plasma cholesterol concentration in male guinea pigs fed the plain diet. Two groups of animals were each fed the plain diet. Both groups received 0.010% w/v ascorbic acid in their drinking water for 1 week. For the following 2 weeks this concentration was increased 10-fold in one group (○—○) and 20-fold in the other group (●—●). In the 4th and 5th weeks the first group continued to receive 0.100% w/v ascorbic acid in their drinking water while in the second group the concentration was increased to 0.400% w/v. Values are the mean±S.E.

TABLE 29. Effect of high levels of ascorbic acid supplementation on plasma cholesterol concentration and on whole blood and liver ascorbic acid concentrations in male guinea pigs fed the plain diet.

	Level of ascorbic acid supplementation given during last two weeks of experiment (% w/v ascorbic acid in drinking water) <sup>a</sup>	
	0.100	0.400
Number of animals	5	5
Plasma cholesterol concentration mg/100ml	55.5±7.06 <sup>b</sup>	56.3±8.27
Ascorbic acid concentration		
Whole blood mg/100ml	1.77±0.079	1.88±0.125
Liver mg/100g	56.6±1.64	63.5±4.77

<sup>a</sup> See legend to Figure 24.

<sup>b</sup> Values are the mean±S.E. Differences between groups, as compared by Student's t-test are not statistically significant.

(b) Discussion.

In the studies by Ginter (1970) it was shown that cholesterol feeding to male guinea pigs for prolonged periods markedly decreased the ascorbic acid concentration in the liver. The second experiment with females confirmed these findings. The lower ascorbic acid concentrations in the blood, liver and adrenals of these animals probably resulted not only from an increased urinary excretion of the vitamin (Ginter, 1970) but also from an

increased demand for ascorbic acid in the catabolism and possibly also the excretion of the additional dietary cholesterol. In guinea pigs fed a 1% cholesterol diet for 10-12 weeks bile acid synthesis and excretion was increased 3-fold (Hansma and Ostwald, 1974). Bile acid synthesis has been shown to depend quantitatively on the availability of ascorbic acid (Ginter et al., 1973b). The biosynthesis of cholesteryl sulphate which is excreted in the faeces, also requires ascorbic acid (Verlangieri and Mumma, 1973).

Thus as ascorbic acid has a stimulatory effect on bile acid synthesis and to some degree on cholesterol excretion, it would be expected that in the cholesterol-fed guinea pig, an increased level of ascorbic acid supplementation would decrease the hypercholesterolaemia proportionately. However the second experiment with females demonstrated an increased plasma cholesterol concentration with increased ascorbic acid supplementation. Although it is difficult to account for this effect, it is certain that the increased plasma cholesterol concentrations were not the result of enhanced cholesterol synthesis. This conclusion follows from two earlier observations. Firstly, a high level of supplementation for 32 weeks to females fed the plain diet had no effect on hepatic and intestinal sterologogenesis. Secondly, sterol synthesis, particularly in the liver, was markedly inhibited in cholesterol-fed guinea pigs (Part V). It is also unlikely that the increased plasma cholesterol concentrations were produced by mobilisation of cholesterol from the tissues. Zaitsev et al. (1964) showed that ascorbic acid supplementation to cholesterol-fed rabbits increased cholesterol deposition in the liver and adrenals.



The increased plasma cholesterol concentrations produced by supplementation with high levels of vitamin C may have resulted from an increase in the absorption of dietary cholesterol. In guinea pigs with chronic latent scurvy the absorption of cholesterol is significantly reduced (Ginter et al., 1969c). This occurs because of a decreased availability of bile acids, fatty acids and monoglycerides. The availability of fatty acids and monoglycerides decreases because the activity of pancreatic lipase is reduced in scorbutic guinea pigs (Ginter et al., 1967). The hypercholesterolaemic effect of high intakes of vitamin C could also have been produced by decreased absorption of copper from the intestine. High levels of ascorbic acid supplementation for several months to male rats has been shown to significantly increase plasma cholesterol concentration (Klevay, 1976). This was attributed to an inhibitory effect of the ascorbic acid on the intestinal absorption of copper, thereby increasing the ratio of zinc to copper ingested. Such an imbalance between zinc and copper had earlier been shown to cause hypercholesterolaemia in rats (Klevay, 1973).

The preceding explanations almost certainly do not fully account for the hypercholesterolaemic effect of the high levels of ascorbic acid supplementation in female guinea pigs because they do not take account of the finding that such supplementation in male guinea pigs has no effect. It is thus necessary to speculate that at least in this species there may be important hormonal influences which determine how ascorbic acid regulates cholesterol metabolism. Ginter and Ondreička (1971) reached a similar

conclusion when they found that chronic latent scurvy significantly increased liver cholesterol concentrations in male but not female guinea pigs. The sex difference in normocholesterolaemic guinea pigs in their cholesterolaemic response to high levels of ascorbic acid supplementation appears not to occur in humans. In a study with equal numbers of normocholesterolaemic men and women, Crawford et al. (1975) showed that supplementation with 1 g of ascorbic acid/day for 4 months had no effect on plasma cholesterol concentrations. However the effect of increasing this level of supplementation was not examined.

As dietary ascorbic acid supplementation does not reduce plasma cholesterol concentration in either the normocholesterolaemic guinea pig or human, it seems that such supplementation particularly with "mega" doses in cases where the plasma cholesterol concentration is already normal, may not be expected to provide any additional benefit in reducing the risk of atherosclerosis. Furthermore, although there is a need for such supplementation in those population groups where chronic latent scurvy is prevalent, this supplementation probably needs only to be made to a level which corresponds to the recommended daily allowance of vitamin C.

The principal conclusions drawn from the work described in this thesis are that in the guinea pig: the low rate of hepatic sterologogenesis is the result of marked feedback inhibition by cholesterol and also possibly by bile acids; feedback inhibition of sterologogenesis by cholesterol occurs not only in the liver but also in the extrahepatic tissues; and intestinal sterologogenesis is probably not regulated by feedback inhibition by bile acids.

It remains to be established whether the marked feedback inhibition of sterologogenesis normally found in guinea pig liver is exerted exclusively by cholesterol or whether a direct feedback effect of bile acids is also involved. On the basis of the findings of Swann *et al.* (1975) it appears that much of the inhibition is exerted by

### CONCLUSION

endogenous cholesterol. Examination of the relative inhibitory effects of lipoproteins from the plasma and intestinal lymph of guinea pigs on hepatic sterologogenesis should provide further information concerning its regulation in guinea pigs.

The finding that in guinea pigs feedback inhibition by cholesterol occurs not only in the liver but also in the intestine and lung is in complete agreement with the findings of Swann *et al.* (1975). Studies with other species, in particular the rat, had shown that only in the liver was sterologogenesis regulated by the cholesterol feedback system (Dietschy and Wilson, 1970a). As a result of the widespread tissue distribution of cholesterol feedback in guinea pigs it would appear that this species may be a more appropriate model for further studies on the identity of the effector involved in feedback inhibition by cholesterol and the



The principal conclusions drawn from the work described in this thesis are that in the guinea pig; the low rate of hepatic sterologogenesis is the result of marked feedback inhibition by cholesterol and also possibly by bile acids; feedback inhibition of sterologogenesis by cholesterol occurs not only in the liver but also in the extrahepatic tissues; and intestinal sterologogenesis is probably not regulated by feedback inhibition by bile acids.

It remains to be established whether the marked feedback inhibition of sterologogenesis normally found in guinea pig liver is exerted exclusively by cholesterol or whether a direct feedback effect of bile acids is also involved. On the basis of the findings of Swann et al. (1975) it appears that much of the inhibition is exerted by endogenous cholesterol. An examination of the relative inhibitory effects of lipoproteins from the plasma and intestinal lymph of guinea pigs on hepatic sterologogenesis should provide further information concerning its regulation in guinea pigs.

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mechanism by which this effector alters the rate of sterologogenesis. It will however first be necessary to establish the identity of the primary rate-limiting enzyme in sterologogenesis in guinea pig tissues. The results of the present studies suggest that the primary rate-limiting enzyme in hepatic and intestinal sterologogenesis in the guinea pig probably occurs at or before HMG CoA reductase. In the liver but not the intestine there also appears to be one or more secondary points of regulation subsequent to the primary rate-limiting step. The identity of the enzyme or enzymes involved remains to be established. Several enzymes subsequent to HMG CoA reductase have been identified as secondary points of regulation in rat liver sterologogenesis (Gould and Swyryd, 1966; Slakey et al., 1972).

Although it has been concluded that intestinal sterologogenesis in guinea pigs is not regulated by feedback inhibition by bile acids, as it is believed to be in other species (Dietschy and Wilson, 1970a), this conclusion is based only on the finding that cholestyramine feeding had either no effect or increased only slightly the rate of sterologogenesis in the ileum. Further studies are needed to substantiate these results. Thus the effects of biliary diversion and of bile acid feeding on intestinal sterologogenesis in guinea pigs should be examined.

In addition to these three main findings the present studies have also shown a marked difference between guinea pigs and rats in the nature and extent of the diurnal rhythms in the rates of hepatic and intestinal sterologogenesis and in feed intake. It is concluded that the guinea pig may be a suitable alternative model to the rat for further

studying the role of the feeding pattern in the regulation of the diurnal rhythms in sterologogenesis. It may be useful, for example, to measure the concentrations, in guinea pigs over a 24-h period, of those hormones which have been shown to regulate the rate of sterologogenesis in rat liver. A comparison of the diurnal variation in the concentrations of these hormones with the variation in feed intake and in hepatic and intestinal sterologogenesis, should provide further information concerning the regulatory effect of feeding on the rate of sterologogenesis. This in turn may help to explain the changes which occur in plasma cholesterol concentration in response to a change in feeding frequency.

In agreement with Swann et al. (1975) the present studies have demonstrated that hepatic sterologogenesis in guinea pigs shows marked individual variation. The individual variation in the rate of sterologogenesis in the liver was much more pronounced than the variation between guinea pigs in plasma cholesterol concentration. This however does not necessarily invalidate the conclusion that the liver is probably the major endogenous source of plasma cholesterol in the guinea pig. It may be that the contribution of exogenous cholesterol to the plasma cholesterol pool in guinea pigs far exceeds that from endogenous sources. In this instance wide individual variation in the overall rate of hepatic sterologogenesis would not be reflected in a similar degree of variation in plasma cholesterol concentration. Apart from this possibility, those guinea pigs with unusually high rates of hepatic sterologogenesis may also show very high rates of bile acid synthesis or high rates of cholesterol excretion. In such animals plasma cholesterol concentration



could thus be maintained at normal levels.

It was apparent from the results of the study on the effect of cholesterol feeding on sterologogenesis in female guinea pigs that guinea pigs differ widely in their ability to metabolise and excrete cholesterol. One animal showed normal plasma and liver cholesterol concentrations and normal rates of sterologogenesis despite an increased dietary cholesterol load which in most animals resulted in enhanced plasma and liver cholesterol concentrations and inhibition of sterologogenesis. It is possible however that guinea pigs which do not accumulate cholesterol in the plasma and tissues when fed cholesterol have a much lower capacity for cholesterol absorption than those animals which do accumulate cholesterol.

Although guinea pigs showed a diurnal variation in hepatic and intestinal sterologogenesis there was no diurnal variation in the concentration of cholesterol in the plasma and tissues. Rhythmic fluctuations in the rate of intestinal sterologogenesis would not have been expected to result in fluctuations in the concentration of cholesterol in the plasma and tissues because only a fraction of the sterol produced by guinea pig intestine is cholesterol (Ockner and Laster, 1966). However as the major sterol produced by guinea pig liver is cholesterol, an increase in the plasma and tissue cholesterol concentrations may have been expected during the dark period when the rate of hepatic sterologogenesis reached a peak. As no such change occurred it appears that guinea pigs may show a diurnal variation in bile acid synthesis which closely parallels the rhythm in sterologogenesis in the liver.

It remains to be established whether guinea pigs fed a diet containing added cholesterol show a diurnal rhythm in plasma cholesterol concentration. As guinea pigs were found to show a diurnal variation in feed intake, the consumption of a diet containing added cholesterol may alter the diurnal stability of plasma cholesterol concentration.

The conclusion of Weight et al. (1974) that ascorbic acid is involved in the regulation of an enzyme subsequent to HMG CoA reductase in the pathway of sterologenesis is supported by the finding in the present studies that there was a significant decrease in the rate of incorporation of labelled mevalonate into sterol by the ileum of guinea pigs in the early stage of acute ascorbic acid deficiency. The identity of the enzyme (or enzymes) involved remains to be established.

Although Björkhem and Kallner (1976) have now clearly shown that cholesterol 7 $\alpha$ -hydroxylase activity is markedly decreased in dietary vitamin C deficiency, the role of ascorbic acid in the regulation of the mitochondrial reactions of bile acid synthesis requires further study. This was not examined in the present studies because most evidence favoured the possibility of an effect on cholesterol 7 $\alpha$ -hydroxylase activity.

It is concluded that dietary vitamin C deficiency probably results in a decrease in the amount of cholesterol entering the body pools not only because of a decrease in the rate of sterologenesis but also because cholesterol absorption is decreased (Ginter et al., 1969c). This effect however is more than counteracted by a decrease in the output of cholesterol resulting from a decrease in bile acid

synthesis. Thus chronic dietary insufficiency of vitamin C results in enhanced plasma and liver cholesterol concentrations (Ginter and Nemec, 1969; Ginter et al., 1969d, 1971a, 1972b, 1973b; Ginter, 1973; Nambisan and Kurup, 1975).

Although it is necessary to add vitamin C to the diets of people with chronic latent scurvy, the addition of vitamin C to the diet at the rate of the recommended daily allowance is sufficient to prevent any increase in plasma cholesterol concentration and to obtain maximal rates of sterol and bile acid synthesis.

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